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Interactions Between IGFBP-3 and Nuclear Receptors in Prostate Cancer Apoptosis

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14. ABSTRACT
IGFBP 3 is a potent inducer of apoptosis in both androgen-dependent and androgen-independent prostate cancer lines. When the nuclear receptor RXRalpha was described as an unexpected intracellular binding partner for IGFBP-3 and effects on DNA transcription were demonstrated, rapid effects of IGFBP-3 on programmed cell death (apoptosis) still could not be explaine These rapid effects on apoptosis were clarified when I hypothesized that IGFBP 3 was a biological signal for Nur77 nuclear receptor translocation to the mitochondria where an apoptotic cascade is initiated. We proposed to determine scientifically the protein regions in each of these important cell death molecules that essential for apoptotic action and demonstrate this observation with mouse models. Our data so far reveal a nuclear export sequence in IGFBP-3. Mutation of this sequence impairs its apoptotic activity. Utilizing the IGFBP 3 KO mouse, we show that IGFBP-3's critical role in castration induced apoptosis. Mating studies are underway to determine the effects of genetically deleting Nur77 and IGFBP-3 in the ontogeny of prostate cancer.

15. SUBJECT TERMS
IGFBP-3, apoptosis, prostate cancer, nuclear receptors

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Introduction

Prostate Cancer (CaP) continues to be the most frequently occurring malignancy (aside from skin cancers), found in American men. IGFBP-3 is a potent inducer of apoptosis in both androgen-dependent and androgen-independent prostate cancer lines. When the nuclear receptor RXRalpha was described as an unexpected intracellular binding partner for IGFBP-3 and effects on DNA transcription were demonstrated, rapid effects of IGFBP-3 on programmed cell death (apoptosis) still could not be explained. These rapid effects on apoptosis were clarified when I hypothesized that IGFBP-3 was a biological signal for Nur77 nuclear receptor translocation to the mitochondria where an apoptotic cascade is initiated. This project will determine scientifically the protein regions in each of these important cell death molecules that essential for apoptotic action and demonstrate this observation with mouse models. The innovative aspects of this grant include: (1) Characterization of a novel interface (i.e. mitochondrial localization) of nuclear receptor / IGFBP superfamilies in the initiation of tumor programmed cell death; (2) Development of pre-clinical mouse models of prostate cancer that can be used to assess therapies that exploit the IGFBP-3:Nur77:RXR cell death pathway; and (3) provide a compelling rationale for Phase I studies of IGFBP-3 (or small molecule mimetics of this pathway) in men with prostate cancer.

Body

Task 1. Characterize IGFBP-3 protein-protein interactions and mitochondrial targeting in vitro and demonstrate that they are essential for IGFBP-3 induced apoptosis.

a. Confirm IGFBP-3/RXR/Nur77 ternary complex formation via protein-protein interaction studies. (Months 1-6)

We have established association as published in our *Carcinogenesis* paper in 2008 and referenced in that year's progress report.

b. Validate a putative nuclear export sequence (NES) in IGFBP-3. (Months 7-9).

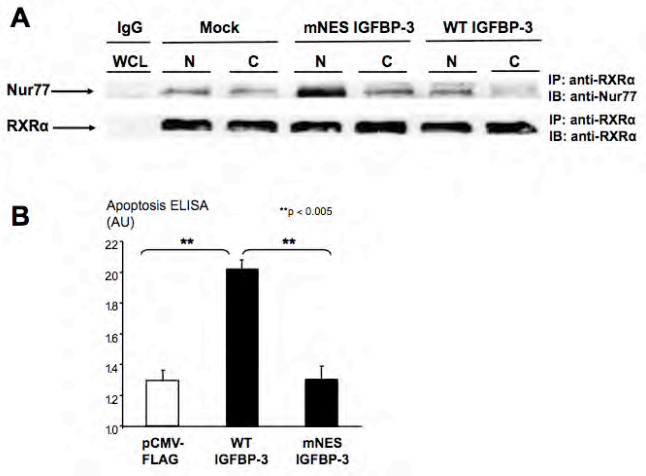

Fig. 1. **Impaired Nuclear Export of IGFBP-3 retains RXRα/Nur77 heterodimers in the nucleus and abolishes the apoptotic actions of IGFBP-3.** (A) Overexpression of mNES IGFBP-3 in 22RV1 cells induces nuclear retention of RXRα/Nur77 protein complexes compared to WT IGFBP-3 overexpression. Mock transfection and co-immunoprecipitation with control IgG are shown on the left. After overexpression, cell lysates were fractionated and immunoprecipitation performed as indicated (B) Transfection of wild-type IGFBP-3 in 22RV1 cells increased oligonucleosomal fragments by 56% compared to empty vector transfection control. Retaining IGFBP-3 in the nucleus by mutating the NES blocked the ability of IGFBP-3 to induce apoptosis.

i. Corroborate a CRM1-dependent export mechanism utilizing the specific inhibitor LMB.

We initiated this experiment several times and unfortunately the CRM1 inhibitor LMB induces massive apoptosis in our system. This was observed in spite of performing careful dose response experiments. We speculate that this may be due to inhibition of nuclear export by nuclear survival factors.

ii. Create NES mutant IGFBP-3 GFP fusion protein and analyze with heterokaryon assay.

We were unable to achieve optimal proximity of human and mouse cell nuclei to assess nuclear export of IGFBP-3 while maintaining visualization on confocal microscopy. This may be a function of our particular system and we did not pursue it further. Secondary to these limitations, we proceeded directly to site-directed mutagenesis.

We have recently published the presence of a NES in IGFBP-3 and validated function by site-specific mutagenesis (Appendix 1). We have demonstrated rapid IGF-independent nuclear localization of IGFBP-3 and cytoplasmic export.

We have previously described nucleo-mitochondrial translocation of nuclear receptor RXRα/Nur77 heterodimers to activate the mitochondrial pathway in apoptosis induction by IGFBP-3. We studied the effect of overexpressing mNES IGFBP-3 on the subcellular localization of RXRα/Nur77. Mutation of the IGFBP-3 NES did not impair interaction with RXRα or Nur77 (data not shown). mNES as well as WT IGFBP-3 expression vector was transiently transfected in 22RV1 CaP cells and cell lysates were fractionated into nuclear and cytoplasmic fractions. Fractions were then

immunoprecipitated with anti-RXRα antibody and immunoblotted with anti-Nur77. Overexpression of mNES IGFBP-3 results in a marked increase in nuclear retention of RXRα/Nur77 (Figure 1A), impairing nuclear export of important mediators of the apoptotic action of IGFBP-3.

To directly examine the functional relationship between the localization of IGFBP-3 and its effects on apoptosis, we transiently transfected 22RV1 prostate cancer cells with expression vector, wild-type (WT) IGFBP-3, and mNES IGFBP-3 (Figure 1B). Transfection of wild-type IGFBP-3 in 22RV1 cells increased oligonucleosomal fragments, as measured by a cell death ELISA, by 56% compared with empty vector transfection control. Retaining IGFBP-3 in the nucleus by mutating the NES completely inhibited the ability of IGFBP-3 to induce apoptosis. These studies confirm the integral role of extranuclear trafficking of IGFBP-3 via its NES for its apoptotic properties.

c. Delineate the mitochondrial targeting sequence (MTS) in IGFBP-3. (Months 7-9).

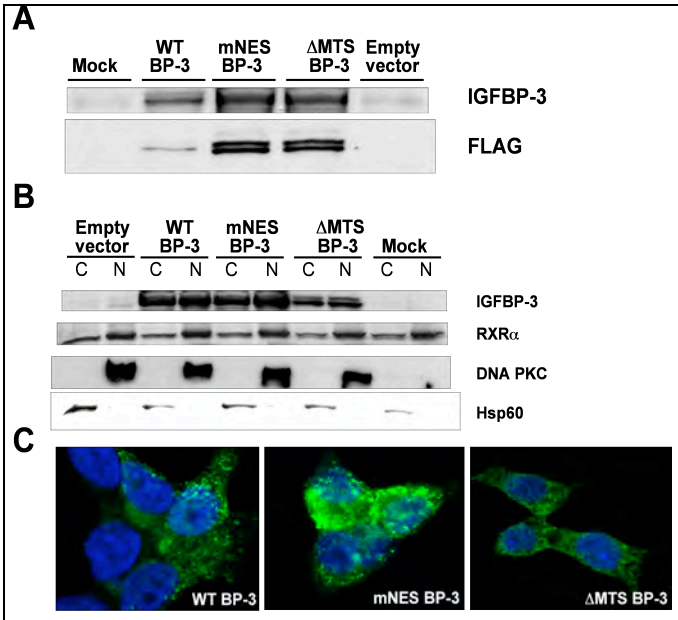

Figure 2. Subcellular localization of IGFBP-3 mutants (A) Recognition of mutant IGFBP-3 by IGFBP-3 antibody. (B) mNES BP-3 accumulates in the nucleus. 22RV1 cells were transfected with WT and mutant IGFBP-3 expression vectors, and fractionated into cytoplasmic and nuclear fractions 48 hours after transfection. BP-3 NES mutant shows increased amounts in nuclear versus cytoplasmic fractions compared to WT IGFBP-3. No known proteolytic sites or N-glycosylation sites in BP-3 are being affected in the Δ MTS mutant. C – cytoplasmic fraction; N – nuclear fraction (C) Immunofluouresence of transfected WT and mutant IGFBP-3 in 22RV1 prostate cancer cells. Green - anti-FLAG (IGFBP-3), blue - DAPI (nuclear staining).

A mitochondrial targeting sequence (MTS) lies within the N-terminal domain of IGFBP-3.

To further support mitochondrial localization of IGFBP-3, we analyzed IGFBP-3's AA sequence with MitoProt (http://psort.ims.u-tokyo.ac.jp/form.html). This calculates the N-terminal protein region that can support a Mitochondrial Targeting Sequence and the cleavage site. The mitochondrial localization of IGFBP-3 is based on hydrophobicity of its leader sequence. This predicts import into the mitochondrial matrix and cleavage between AA13 and 14.

We constructed a mutant NES IGFBP-3:FLAG (C-terminal) fusion consisting of leucine to alanine conversions at residues 197 and 200, since analogous mutations in other NES-containing proteins have been reported to prevent nuclear export. Wild-type or mutant NES:FLAG constructs were cloned in expression vectors; verified by sequencing; and transiently transfected to assess subcellular localization. In addition, the ΔMTS mutant was generated by deletion of the MTS between AA 27 and 40 with preservation of the signal peptide.

Western immunoblotting revealed that anti-IGFBP-3 antibody recognized the mutant NES as well as the ΔMTS protein (Fig 2A). Fractionation of transfected 22RV1 prostate cancer cells into nuclear and cytoplasmic fractions revealed that whereas WT IGFBP-3 had equal distribution between nuclear and cytoplasmic fractions after 48 hours transfection, BP-3 NES mutant shows increased amounts in nuclear versus cytoplasmic fractions compared to WT IGFBP-3 (Fig 2B). Immunoblotting with DNA PKC and Hsp60 were used to assess purity of the nuclear and cytoplasmic fractions respectively. No significant change in subcellular distribution as assessed by these fractionation methods was noted in the ΔMTS IGFBP-3 mutant.

Analysis by indirect immunofluoresencent confocal microscopy correlated with subcellular subfractionation for IGFBP-3 localization with known mitochondria/ER markers (Fig 2C). No known proteolytic sites or N - glycosylation sites in BP-3 are being affected in the Δ MTS mutant.

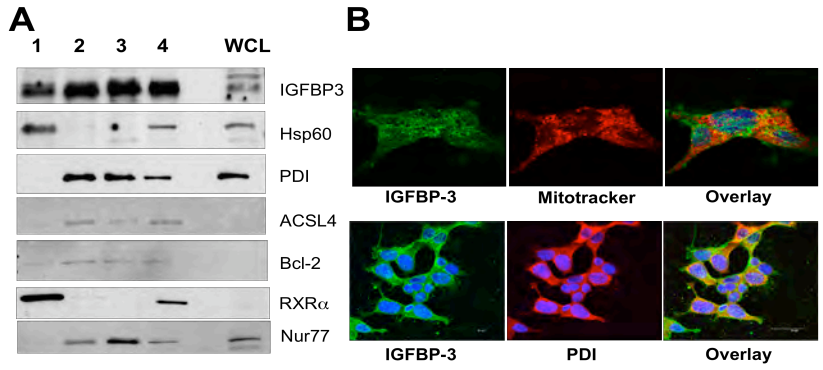

Figure 3. Endogenous Mitochondria and ER Localization of IGFBP-3. (A) 22RV1 prostate cancer cells were fractionated into Lane 1- Mitochondrial Fraction; Lane 2–MAM (Mitochondria Associated Membranes); Lane 3-ER (Endoplasmic Reticulum); and Lane 4–Pellet from MAM. (b) Co-localization (yellow) of IGFBP-3 (green) with Mitochondrial (Mitotracker - red) and ER markers (PDI - red) by immunofluouresence confocal microscopy.

Association of IGFBP-3 with Mitochondria and Endoplasmic Reticulum *in vitro.* In an attempt to obtain more detailed information on IGFBP-3 subcellular protein localization of endogenous IGFBP-3 in 22RV1 prostate cancer cells, we turned to subcellular fractionation via a protocol that combines differential and Percoll gradient centrifugations as this is the preferred method for higher purity fractions. The internal membranes were segregated into mitochondria (Fig 3A, Lane 1), mitochondria-associated membrane (MAM; Lane 2), endoplasmic reticulum (ER; Lane 3), and a pellet from the MAM fraction that was collected at low *g* centrifugation, representing an intermediate zone between mitochondria and MAM fraction (Lane 4). The MAM fraction, a subdomain of the ER, which consists of membrane tubules that provide direct physical contact between the ER and mitochondria, was fractionated to high purity. The presence of IGFBP-3 in the various membrane fractions was assessed by immunoblotting. The relative purity of the fractions was assesed by the presence of specific marker antibodies (Hsp60 – mitochondria; PDI – ER, ACSL4 – MAM). Under baseline conditions *in vitro* IGFBP-3 localizes to the mitochondria (Lane 1), and is even more abundantly represented in the ER and MAM membrane fractions (Lane 2 and 3 respectively).

Bcl-2 expression is detected in all membrane fractions as well as has been previously

described. RXRα was detected most prominently in the mitochondria fraction, as well as a faint presence in the MAM. RXRα presence in Lane 4 likely represents mitochondria in this transitional layer. In addition, another binding partner of IGFBP-3, Nur77 was also identified in the MAM and ER, with faint presence in the mitochondrial fraction. Mitochondrial localization of RXRα and Nur77 has been described previously. Immunoflourescence studies revealed co-localization of endogenous IGFBP-3 with Mitotracker and PDI, an ER marker, consistent with the subcellular fractionation studies (Fig 3B).

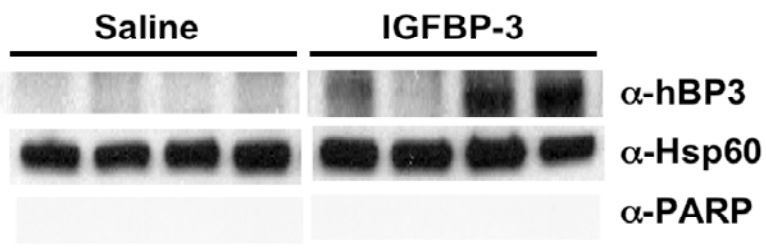

Figure 4. Prostate Cancer Xenografts Internalize recombinant human IGFBP-3 (rhIGFBP-3) which localizes to mitochondria *in vivo*. 22RV1 CaP xenografts on SCID male mice were treated with IGFBP-3, mitochondria were isolated, and immunoblotted for IGFBP-3.

Mitochondrial targeting of IGFBP-3 *in vivo.* To determine whether administered IGFBP-3 is targeted to mitochondria *in vivo*, we administered saline or recombinant human IGFBP-3 (Insmed, Inc.) at 40 mg/kg IP divided twice a day for 3 days to male SCID mice carrying 22RV1 prostate cancer tumors. Over this short time period, tumor size did not decrease (data not shown) as we have described for a longer incubation, and as others have described in a transgenic model utilizing a non-IGF binding mutant (Cohen 2006; Silha et al. 2006).

Mitochondria were isolated utilizing the Sigma animal tissue mitochondrial isolation kit and associated proteins were resolved by SDS-PAGE (Figure 4). In 3 of 4 tumors examined, IGFBP-3 was found associated with mitochondria. Hsp60 and PARP were used to assess purity of the mitochondrial fraction.

d. Assess the effects of mutant NES IGFBP-3) on apoptosis. (Months 9-12)

We have begun to assess the effects of the NES mutant on apoptosis and show that mutation prevents efficient apoptosis by IGFBP-3. (Fig. 1B)

Task 2. Define the role of the IGFBP-3/RXR/Nur77 apoptotic pathway in vivo in the TRAMP mouse model.

a. We will age the Nur77 KO and IGFBP-3 KO mice to determine if and when these mice develop prostatic pre-neoplastic lesions. (Months 1-18)

No spontaneous prostate tumor development was observed in IGFBP-3KO mice; however, we did observe splenic lymphomas in 30% of female IGFBP-3KO mice, but not Wild-Type (WT), at 80 weeks of age (data not shown).. We did not observe spontaneous prostate cancer tumor development in the Nur77 KO mice with aging (data not shown).

b. Examine the role of IGFBP-3 in apoptosis induced by androgen withdrawal by castration of TRAMP and IGFBP-3 KO:TRAMP mice

i. Develop IGFBP-3 KO:TRAMP cross and assess mouse aging and tumor chronomics. (Months 1-24). Total 100 mice.

We quickly discovered that the TRAMP model was a very aggressive, early metastatic mouse model of CaP and did not see any difference with the IGFBPKO mating. Therefore, we chose to study a slower developing model, the Myc model of prostate cancer (expressing human c-*Myc* driven by a prostate specific promoter) developed at UCLA.

ii. Examine subcellular localization of RXR, IGFBP-3, and Nur77 utilizing *in situ* immunohistochemistry and immunoblot post cellular fractionation in tumors before and after castration (25 mice/group; 13 castration and 12 "sham" castration) at 12 weeks of age (Total 75 mice). Animals to be sacrificed after 6h (2 mice/group) and then every 24h for 4 days. (months 1-6)

After initial immunohistochemistry staining of tissue with RXR and Nur77, we discovered that the available antibodies are not very specific with multiple banding patterns on Western blot. Therefore, we will await the availability of more specific commercial antibodies.

iii. Evaluate apoptosis utilizing TUNEL staining and evaluate protein subcellular distribution of IGFBP-3, RXR, and Nur77 by Western blotting. (Months 6-12) (**Fig. 5**).

WT mice showed a dramatic, 6-fold, increase in the number of TUNEL-positive nuclei at 48 hours post castration. However, IGFBP-3 KO mice prostates failed to show any significant increase in TUNEL staining at 48 hours. By 72 hours TUNEL staining returned to near baseline levels in WT mice and remained near baseline levels in IGFBP-3 KO mice. Serum IGFBP-3 levels were undetectable in the KO mouse and remained unchanged in WT mice post castration.

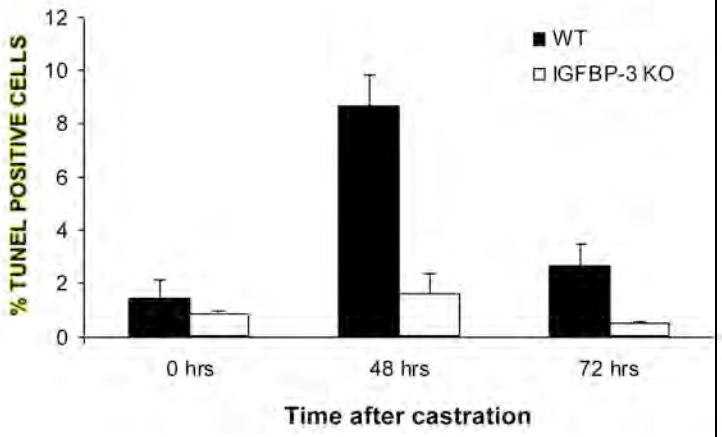

Fig. 5. IGFBP-3 is essential for androgen deprivation induced apoptosis. Twelve-week-old male wild type (WT) mice and IGFBP-3 KO mice were surgically castrated. Prostates were harvested after 48 and 72 hours. Prostates and testes were fixed and stained with H&E for morphologic analysis and quantification of apoptosis using a TUNEL strategy. Apoptosis was quantified by counting the number of TUNEL-positive cells.

p53 has been shown to be required for prostatic apoptosis, and we have now shown that IGFBP-3, which is activated downstream of p53, is also required for this process. In summary, this is the first description of an *in vivo* role for IGFBP-3 in physiological cell death and indicates that IGFBP-3 is critical for prostatic apoptosis, a fact with potential therapeutic implications in prostate cancer

Increased metastasis in IGFBP-3KO:Myc mice

When IGFBP-3KO:Myc mice were necropsied at 80 weeks of age, prostate tumors tended to be larger in the IGFBP-3KO:Myc mice when compared to WT. In addition, we identified lung metastases in 55% of IGFBP-3KO:Myc mice, while none of the WT:Myc mice exhibited any metastases (Figure 6A, 6B).

This is a remarkable finding, as metastases in the Myc model of prostate cancer have not been described to date, and suggests that a major anti-tumorigenic role of endogenous IGFBP-3 is as a metastasis suppressor.

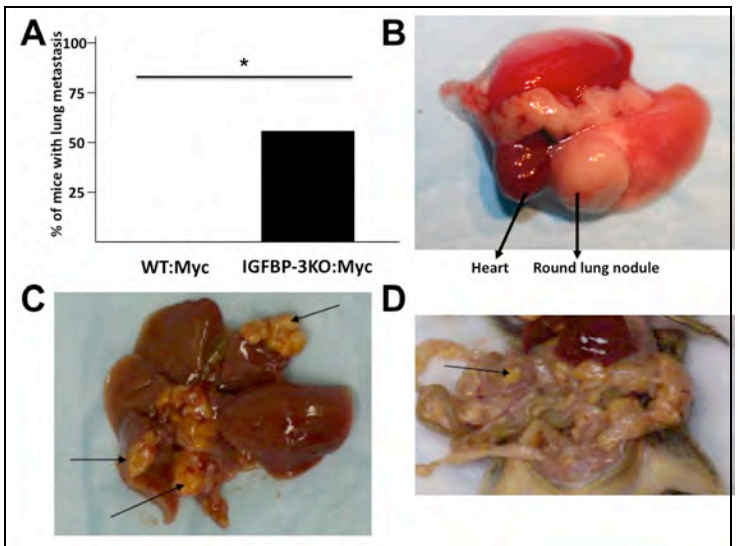

Fig. 6. (A) Increased metastasis in IGFBP-3KO:Myc mice. Incidence of lung metastases in 80-week-old WT:Myc (n=22) and IGFBP-3KO:Myc (n=18) male mice. *p=0.01 by Chi Square. (B) Representative photograph of lung nodule. (C) Multiple metastases in liver (arrows). (D) Multiple metastases in intestine (arrow pointing at one of many).

We characterized the presumptive lung metastases by immunohistochemical staining for Clara Cell 10 (CC10) protein, a marker of bronchiolar epithelial cells. CC10 is expressed in the lung in non-ciliated airway epithelial cells and in urogenital secret ions and is not expressed in prostate cells. Most primary lung adenomas and adenocarcinomas are of Clara cell origin.

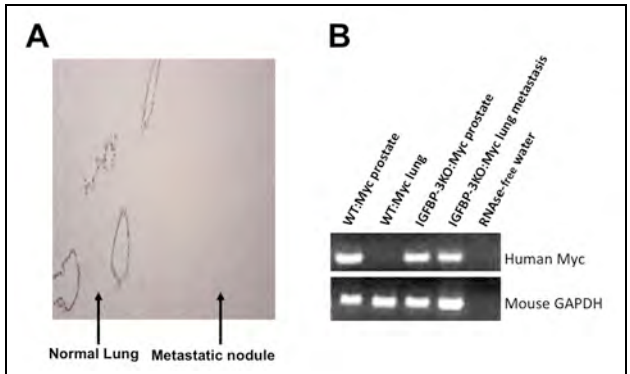

Fig. 7. (A) CC10 immunohistochemical staining of a representative lung nodule and adjacent normal lung tissue in a IGFBP-3KO:Myc mouse. (B) RT-PCR of human Myc of primary tissue RNA from mouse necropsy confirming metastatic nature of lung nodules.

As shown in Figure 7A, normal lung parenchyma stains well for CC10, and the lung nodule does not, showing that the observed lung nodules are not of lung derivation. Occasionally, some mice developed metastatic nodules in visceral organs including liver and intestine (Figure 6C, 6D). RT-PCR analysis for the human *Myc* gene verified expression in the lung nodules, thereby confirming their metastatic nature (Figure 7B).

Establishment and characterization of prostatic epithelial cell lines from the WT:Myc and IGFBP-3KO:Myc mouse models

To facilitate our investigation of the molecular mechanisms underlying IGFBP-3-controlled prostate tumorigenesis and metastasis, we isolated primary prostatic cells from WT:Myc and IGFBP-3KO:Myc *mice.* After serial passages and spontaneous immortalization, several clonally derived cell lines were established and from which two lines each were characterized and studied herein. A representative cell line of each is

presented.

To determine the properties of these established cell lines, we performed Western blot analysis using antibodies corresponding to phenotypic markers for cell types in the prostate epithelium.

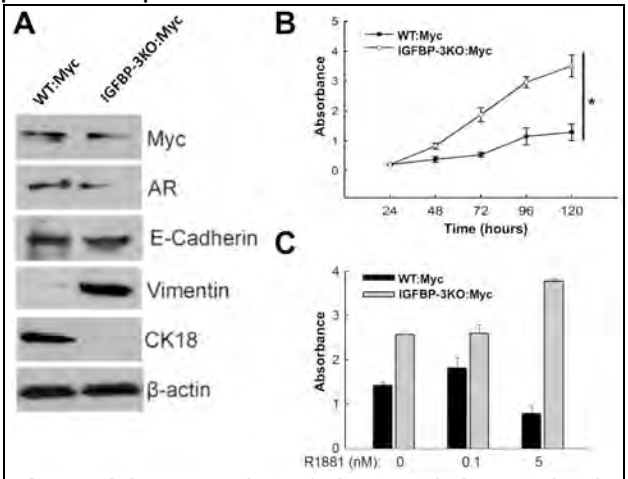

Fig. 8. (A) Expression of characteristic proteins in primary WT:Myc and IGFBP-3KO:Myc cell lines by Western immunoblot. (B) Cell proliferation curves of WT:Myc and IGFBP-3KO:Myc cell lines by MTT assay (C) Differential response to androgen by WT:Myc and IGFBP-3KO:Myc cell lines.

As shown in Figure 8A, both WT:Myc and IGFBP-3KO:Myc cell lines maintained androgen receptor (AR) expression, but the IGFBP-3KO:Myc cell line exhibits the loss of CK8, a luminal epithelial marker. Protein expression of E-cadherin did not change; however, another well-known marker of epithelial-mesenchymal transition vimentin, was significantly induced in the IGFBP-3KO:Myc cell line. This expression profile is similar to that seen in the metastases of a human CaP xenograft-mouse model with knockdown of endogenous DAB2IP (member of the Ras GTPase-activating protein family). Loss of DAB2IP facilities epithelial-mesenchymal transition (EMT) leading to CaP metastasis.

To measure the biological effects of *IGFBP-3* gene deletion in *Myc CaP* cells, we first compared cell growth properties of the WT:Myc and IGFBP-3KO:Myc cell lines. As shown in Figure 8B, cell proliferation rates were significantly increased to over 3-fold at 120 hours in the IGFBP-3KO:Myc cell line as compared to WT:Myc cells**,** suggesting that IGFBP-3 plays a role in the cell cycle progression of *Myc* prostatic epithelial cells.

Additionally, in the absence of the androgen R1881, WT:Myc cells proliferated slower than IGFBP-3KO:Myc cells (Figure 8C). At lower concentrations (0.1 nM), R1881 stimulated WT:Myc cell growth while IGFBP-3KO:Myc cells were not significantly stimulated. When a high concentration of R1881 was used (5 nM), WT:Myc cells growth is inhibited but not IGFBP-3KO:Myc cells. Therefore, genetic loss of IGFBP-3 results in a differential growth response to androgen.

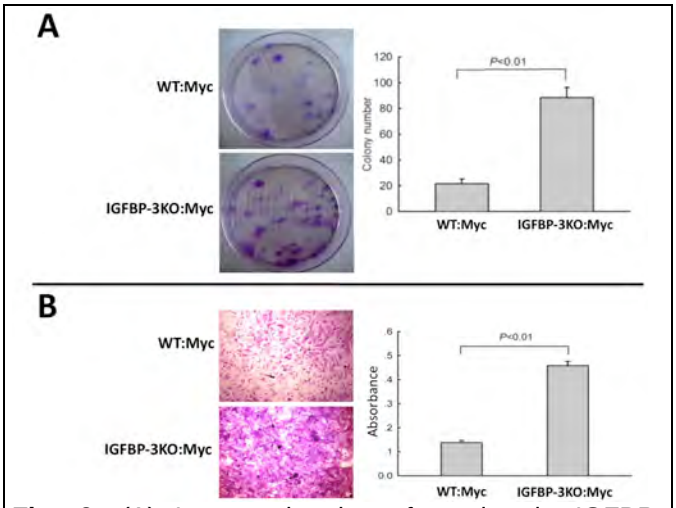

Fig. 9. (A) Increased colony formation in IGFBP-3KO:Myc cells (B) Increased invasiveness of IGFBP-3KO:Myc cells

IGFBP-3 **loss leads to increased colony formation and invasiveness of CaP cells *in vitro***

To determine whether *genetic*

deletion of IGFBP-3 leads to enhanced transformation potential, we compared the colony-forming efficiency of WT:Myc and IGFBP-3KO:Myc cell lines. After 14 days in culture, colonies were photographed and counted. IGFBP-3KO:Myc cells formed over 4-fold more colonies when compared to WT:Myc cells (Figure 9A).

In addition, to evaluate the relation between cell migratory ability and the expression of IGFBP-3, we conducted a migration assay using a modified Boyden chamber method. The invasiveness of IGFBP-3KO:Myc cells when compared to WT:Myc cells was increased over 3-fold (Figure 9B). Taken together, these data support a role for IGFBP-3 in metastasis suppression in prostate cancer.

c. Study the *in vivo* effects of IGFBP-3 replacement treatment in the IGFBP-3 KO:TRAMP model of prostate cancer.

Unfortunately, our supplier of recombinant human IGFBP-3 has discontinued production of peptide for the time being secondary to financial constraints. However, we are hopeful that they will resume production within the year to obtain material for the in vivo replacement study.

i. Comparison of response to a 4-week course of IGFBP-3 treatment in the TRAMP and TRAMP/IGFBP-3 KO mice on tumor size and histology. (Months 24-30) 7 mice/group total 28 mice (including controls).
ii. Evaluation of tumor apoptosis by TUNEL staining and proliferation by PCNA staining. (Months 24-30)
iii. Perform immunohistochemistry for subcellular localization of IGFBP-3, Nur77, and RXR as well as subcellular fractionation and immunoblotting for IGFBP-3, RXR, and Nur77. (Months 30-36)

Key Research Accomplishments

- Defined a Nuclear Export Sequence in IGFBP-3
- Created NES / MTS mutants of IGFBP-3
- Established Mitochondria and Endoplasmic Reticulum Localization of IGFBP-3 in vitro
- Demonstrated Mitochondrial Localization of Recombinant Administered IGFBP-3 to prostate cancer xenografts in vivo.
- Assessed Mutant Effects on IGFBP-3 induced apoptosis
- Castrated IGFBP-3 KO and WT mice
- Aged IGFBP-3 KO and WT mice and assessed for CaP incidence
- Demonstrated that IGFBP-3 is essential for androgen deprivation-induced apoptosis
- Developed IGFBP-3KO:Myc cross
- Aged WT:Myc and IGFBP-3KO:Myc cohort.
- Demonstrated increased metastasis in IGFBP-3KO:Myc cohort and determined that metastasis was of prostatic origin
- Developed primary cell lines from prostatic tumors in WT:Myc and IGFBP-3KO:Myc mice and performed initial characterization.

Reportable Outcomes

Manuscripts Published

1. Yamada PM, **Lee KW.** 2009. Perspectives in Mammalian IGFBP-3 Biology: Local vs. Systemic Action. Am J Physiol Cell Physiol. May;296(5):C954-76. Epub 2009 Mar 11. PMCID: 19279229 (Appendix 1)
2. Paharkova-Vatchkova, V, **Lee KW.** 2010. Nuclear Export and Mitochondrial and ER-targeting of IGFBP-3 regulates its apoptotic properties. 2010 Mar 8;17(2):293-302. *Endocrine-Related Cancer*. PMCID: 20228135. (Appendix 2)
3. Jia Y, **Lee KW**, Swerdloff RS, Hwang D, Cobb LJ, Hikim AS, Lue YH, Cohen P, and Wang C. 2010. The interaction of insulin-like growth factor binding protein-3 and bax in mitochondria promotes male germ cell apoptosis. *J Biol Chem*. **285**:1726-32. PMID: 19887447
4. Lue Y, Swerdloff R, Liu Q, Mehta H, Sinha Hikim A, **Lee KW**, Jia Y, Hwang D, Cobb LJ, Cohen P, Wang C. 2010. Opposing Roles of Insulin-Like Growth Factor Binding Protein 3 and Humanin in the Regulation of Testicular Germ Cell Apoptosis. *Endocrinology*. **151**:350-7. PMID: 19952275

Manuscripts Submitted

1. Mehta HH, Gao Q, Galet C, Wan J, Said J, Sohn JJ, Lawson G, Cohen P, Cobb LJ, **Lee KW.** 2010. IGFBP-3 is a Metastasis Suppression Gene in Prostate Cancer. *Cancer Cell*

Poster Presentations

Mehta HH, Hwang DL, Sohn JJ, Lue YH, Wang C, Said J, Cohen P, **Lee KW**. IGFBP-3 is essential for prostate apoptosis induced by androgen deprivation. Endocrine Society. San Francisco, CA. June 2008.

Paharkova-Vatchkova V, **Lee KW**. Identification of Nuclear Export Sequence (NES) within IGFBP-3 and its Contribution to Apoptosis. Endocrine Society. San Francisco, CA. June 2008.

Oral Presentations

Lee KW. Characterization of a putative “BH3-like” domain establishes IGFBP-3 as a unique intrinsic pathway activator that integrates cell survival and death signals in the mitochondrial regulatory network. Fifth International Congress of the GRS and IGF Society. New York, NY. October 2010.

Conclusions

Thus, we conclude that IGFBP-3 is a potent apoptosis inducer with potential implications in prostate cancer. IGFBP-3 induces apoptosis of both androgen-dependent and –independent CaP in vitro, and this has recently been demonstrated in vivo. On a cell biology level, to my knowledge IGFBP-3 is the only molecule known with an endocrine (serum carrier for IGF), as well as an auto-/paracrine function (that can be IGF-independent) with nuclear and extranuclear functions. Therefore, the proposed work shifts the current thinking of IGFBP biology. We have begun to characterize how subcellular localization of IGFBP-3 effects apoptosis induction. In addition, we have for the first time implicated IGFBP-3 in physiologic apoptosis induced by androgen deprivation utilizing the IGFBP-3 KO mouse. Practically speaking, the mechanistic work proposed therein represents the foundation for a new therapeutic intervention in the treatment of men with prostate cancer. These experiments will provide a research-based rationale for clinical trials of IGFBP-3 and establish a role for such therapy in androgen-dependent and –independent prostate cancer. IGFBP-3 has recently undergone successful phase 1 studies in humans and is about to enter phase 2 studies in cancer patients. If successful, these expected findings will improve our understanding of this emerging prostate cancer therapy and facilitate further clinical development in men with prostate cancer.

References

- Liu B, **Lee KW**, Anzo M, Zhang B, Zi X, Tao Y, Shiri L, Pollak M, Lin S, Cohen P. 2007. IGFBP-3 inhibition of prostate cancer progression involves suppression of angiogenesis. 26:1811-9. *Oncogene*.

- **Lee KW**, Ma L, Cobb L, Paharkova-Vatchkova V, Liu B, Milbrandt J, Cohen P. 2007. Contribution of the Orphan Nuclear Receptor Nur77 to the Apoptotic Action of IGFBP-3. *Carcinogenesis* 28:1653-1658.

- Paharkova-Vatchkova, V, **Lee KW.** 2009. Nuclear Export and Mitochondrial and ER-targeting of IGFBP-3 regulates its apoptotic properties. In Press. *Endocrine-Related Cancer*. (Appendix 1)

- Yamada PM, **Lee KW.** 2009. Perspectives in Mammalian IGFBP-3 Biology: Local vs. Systemic Action. Am J Physiol Cell Physiol. May;296(5):C954-76. Epub 2009 Mar 11. PMCID: 19279229 (Appendix 2)

Perspectives in mammalian IGFBP-3 biology: local vs. systemic action

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Perspectives in mammalian IGFBP-3 biology: local vs. systemic action

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Yamada PM, Lee K. Perspectives in mammalian IGFBP-3 biology: local vs. systemic action. *Am J Physiol Cell Physiol* 296: C954–C976, 2009. First published March 11, 2009; doi:10.1152/ajpcell.00598.2008.—Insulin-like growth factor (IGF) binding protein (IGFBP)-3 has traditionally been defined by its role as a binding protein and its association with IGF delivery and availability. Development of non-IGF binding IGFBP-3 analogs and the use of cell lines devoid of type 1 IGF receptors (IGF-R) have led to critical advances in the field of IGFBP-3 biology. These studies show that IGFBP-3 has IGF-independent roles in inhibiting cell proliferation in cancer cell lines. Nuclear transcription factor, retinoid X receptor (RXR)-α, and IGFBP-3 functionally interact to reduce prostate tumor growth and prostate-specific antigen in vivo. Moreover, IGFBP-3 inhibits insulin-stimulated glucose uptake into adipocytes independent of IGF. The purpose of this review is to highlight IGFBP-3 as a novel effector molecule and not just another "binding protein" by discussing its IGF-independent actions on metabolism and cell growth. Although this review presents studies that assume the role of IGFBP-3 as either an endocrine or autocrine/paracrine molecule, these systems may not exist as distinct entities, justifying the examination of IGFBP-3 in an integrated model. Also, we provide an overview of factors that regulate IGFBP-3 availability, including its production, methylation, and ubiquitination. We conclude with the role of IGFBP-3 in whole body systems and possible future applications of IGFBP-3 in physiology.

insulin-like growth factor; nonsuppressible insulin-like activity; insulin-like growth factor binding protein-3

INSULIN-LIKE GROWTH FACTOR (IGF) was originally named nonsuppressible insulin-like activity (NSILA) because it stimulated glucose uptake into adipocytes, sharing "insulin-like" activity (81). Whereas anti-insulin antibodies were capable of abrogating the hypoglycemic effects of insulin, they did not abolish the effects of NSILA, hence, the term "nonsuppressible" was incorporated into its name. The term "NSILA" was renamed "somatomedin" in 1972 to describe its hormonal relationship to growth hormone (51).

Soon after the characterization and quantification of NSILA in rat serum (229), tracer technology identified that NSILA-S (serum NSILA) had a carrier protein (132). This was demonstrated by measuring the half-life of NSILA after the infusion of ^{125}I-labeled NSILA-S (IGF) or ^{125}I-labeled NSILA-S plus NSILA-S into rats. NSILA-S of low-molecular weight (~7.5 kDa) was cleared 20 min postinjection, whereas the high-molecular weight (~100 kDa) persisted for 3 h. Chromatography identified that the ~100-kDa complex contained NSILA-S and its binding protein. The described low- and high-molecular weights (265) presumably corresponded to the molecular weights of IGF-1 and -2 alone (7.5–7.6 kDa) and the IGF-ALS-IGFBP-3 heterotrimeric complex (150 kDa), respectively. Today it is well known that IGF complexes with a leucine-rich acid labile subunit (ALS), and insulin-like growth factor binding protein (IGFBP-3), the most abundant circulating binding protein. Binding amplifies the half-life of IGF up to 12–15 h (102).

Perfusion of human serum into isolated rat hearts showed that glucose uptake remained unchanged (174), despite reports that free NSILA has insulin-like, hypoglycemic effects (173). This suggested that the insulin-like activity of partially purified NSILA on the rat heart was abolished in the presence of the NSILA-carrier protein complex (174). ^{125}I-labeled NSILA-S action was inhibited by its carrier protein, and the size of the NSILA-S-carrier protein complex prevented IGF efflux from vasculature. In 1976, NSILA was sequenced, revealing that it shared 48% homology with human proinsulin (203) and was renamed IGF-1. In 1989, this transporter of 70–90% of all circulating IGF was officially termed "insulin-like growth factor binding protein-3" (IGFBP-3) [Workshop on IGF Binding Proteins, Vancouver, Canada, 1989 (18)].

Presumably IGFBP-3 is produced and released by hepatic Kupffer and endothelial cells (264) into the systemic circulation to affect auxological growth via endocrine regulation. The majority of in vitro studies that have demonstrated IGF-independent actions of IGFBP-3 on cell growth have been performed in culture and therefore simulates autocrine/paracrine regulation. Although this review presents studies that use IGFBP-3 in an endocrine (as an IGF transporter and binding protein) or autocrine/paracrine system (local release of IGFBP-3 from tissues to be reinternalized by the original cell or by neighboring tissues), these systems may not exist as distinct models. This may justify the examination of IGFBP-3 in an integrated model. Figure 1 illustrates a separated view of local and systemic regulation.

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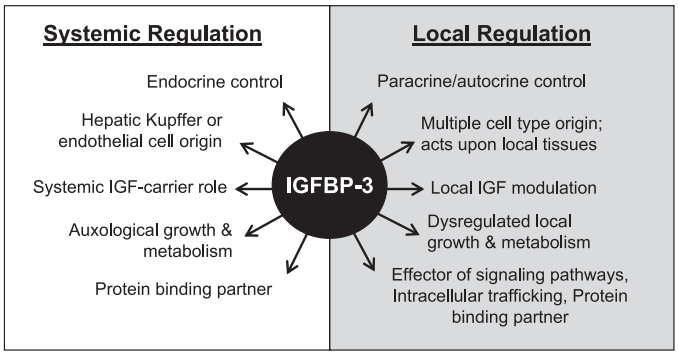

Fig. 1. Local and systemic actions of insulin-like growth factor binding protein-3 (IGFBP-3). The majority of studies employ models that represent either systemic or local regulation and have not incorporated both systems into their model of study. Whether interaction between these systems has physiological significance is unknown.

IGFPB-3 Across Phylogeny

IGFs and IGFBP-3 have been extensively studied in humans, mice (177), and rats (180). The IGF system and its binding proteins have also been examined in a variety of species including cow (88), pig (11), water buffalo (124), cattle (238), sheep (86), horse (75), dog (69), wallaby (33), kangaroo (258), tilapia (190), and zebrafish (157). Of particular interest, a secreted 27-kDa protein that binds to proteins of the insulin superfamily of insect cells (S9) was purified, cloned, and characterized in 2000 (225). This protein was shown to inhibit insulin activation of the insulin receptor and bound to IGF-I, -II, pro-insulin, and mini-proinsulin with high affinity. Eight years later, Imp-L2, the functional equivalent of IGFBP in *Drosophila* was identified in vivo, which is the first documentation of an insect insulin/IGF binding protein (114). Imp-L2 was shown to bind to and antagonize *Drosophila* insulin-like peptide 2 (Dilp2). Imp-L2 overexpression reduced body size, whereas loss of Imp-L2 resulted in increased body size, similar to the phenotypes of the transgenic and knockout IGFBP-3 mice (221, 253). These observations indicated that Imp-L2 is an IGFBP equivalent. In addition, zebrafish have also been used to study the role of IGFBP-3 in growth and development (157). Knockdown of IGFBP-3 delayed pharyngeal skeleton morphogenesis, reduced pharyngeal cartilage differentiation, decreased inner ear size, and disrupted semicircular canal formation in embryos; these effects were rescued with IGFBP-3 expression. IGF binding protein conservation in invertebrates portends a significant physiological role across phylogenies.

Unraveling IGF-Independent Actions

The term IGFBP-3 does not fully describe its roles because it not only functions as a binding partner, but it also has important functions not involving IGF. IGFBP-3 has historically been described as a mediator of endocrine cell growth through its IGF-dependent effects. The function of IGFBP-3 was viewed as a binding protein of IGF-1 to either transport IGF-1 to its receptor, enhancing IGF-1 actions, or sequester IGF-1 from its receptor, inhibiting its action. Advances in the field have enabled researchers to demonstrate evidence for IGF-independent effects of IGFBP-3 using various strategies: *1*) IGFBP-3 mutants that do not bind to IGF-1 (26, 115, 257) or IGF-1 analogs with reduced affinity for IGFBP-3 [long R3-IGF-1, GroPep, Australia, and des-(1-3)-IGF-1] (254, 255), *2*) 16- and 22/25-kDa IGFBP-3 fragments with total and partial loss of IGF affinity, respectively (143), *3*) transfection of the IGFBP-3 vector into cells lacking IGF-R (197, 236), and *4*) use of IGF-1 negative cell lines (breast cancer cells, chrondrocytes) (97, 232). Identification of putative IGFBP-3 cell surface receptors and binding to these receptors with specificity and high affinity have suggested IGFBP-3 is capable of IGF-independent actions (184, 186, 256).

Structure

Human IGFBP-3 is composed of 264 amino acids and contains 3 functional domains: a nonconserved central domain and highly conserved cysteine-rich carboxyl (COOH)- and amino (NH_2)-domains. The molecular weight of IGFBP-3 would be 29 kDa based on its amino acid sequence; however, its actual molecular weight ranges from 46 to 53 kDa secondary to potential glycosylation at three different NH_2-linked sites (247). When proteins are separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), IGFBP-3 is detected as a doublet at 42/45 kDa.

Residues 1–87 and 184–264 comprise the NH_2 and COOH domains, respectively. The nonconserved domain spans residues 88–183 or 88–148, with or without the heparin-binding domains (HBD), respectively (256). Another HBD is found in the COOH-terminal, residues 241–259 (90). IGF binding occurs at both NH_2 and COOH terminals (5, 84), and the NH_2-terminal region, including Ile^{56}, Leu^{80}, Leu^{81}, contributes to a hydrophobic pocket that is important for IGF-1 binding (26). Many molecular interactions are localized to the COOH-terminal sequence 215–232. For example, residues 228–232 at the COOH terminal are required for ALS, IGF-1, and heparin binding (17, 73). Also contained within the important residues 215–232 in the COOH-terminal domain is a nuclear localization sequence (NLS) responsible for translocation into the nucleus through nuclear transport factor importin-β (210). Residues within the NH_2 terminal and motifs within the COOH terminal are involved with interaction with nuclear partners RXRα and retinoic acid receptor (RARα) (209). A metal-binding domain (MBD) is also contained in the COOH terminal, which spans the NLS and a caveolin-binding sequence (223). Of the six IGFBPs, IGFBP-3 and IGFBP-5 are most similar in structure, where both contain similar functional domains comprising: *1*) the IGF binding region of the NH_2 and COOH terminus (23, 84, 129), *2*) heparin-binding motifs in the central domain (77, 230), *3*) functional transactivation domain in the NH_2 terminal (261), and *4*) NLS (210), heparin-binding (21), ALS-binding (73, 235), and cell binding domains (73, 235) in the COOH terminal. Furthermore, since type 1α collagen has been shown to physically interact at a region in close proximity to the NLS domain, binding interaction may ultimately affect cell adhesion and migration (162).

Cell Surface Binding Partners and Internalization

IGFBP-3 contains glycosaminoglycan-binding domains, allowing IGFBP-3 to localize to the extracellular matrix or cellular surfaces. IGFBP-3 attachment to the matrix or cell surfaces decreases the affinity of IGFBP-3 for IGF-1, enhancing IGF action. Glycosaminoglycans, heparin, and heparin

sulfate bind to the COOH terminal of IGFBP-3 (77). Like the COOH terminal domain, the central domain also contains two heparin-binding consensus sequences, which are unmasked only when the COOH terminal domain has been deleted (70, 77). Additional glycosaminoglycans have been shown to bind to IGFBP-3. For example, dermatan sulfate was shown to bind to either domain by inhibiting heparin binding. Dermatan sulfate is found in blood vessels and heart valves and may have a role in coagulation and wound repair (77). Heparin binding was inhibited with lower efficacy by chondroitin sulfate A, a structural component of cartilage, and hyaluronic acid, a major component of extracellular matrix (ECM) (77). IGFBP-3 contains essential glycosaminoglycan binding sequences that not only bind heparin and heparan sulfate, but this critical sequence also binds to the ECM and cell surfaces (21).

IGFBP-3 binds to fibrinogen and fibrin (29). Similar to the IGFBP-3-plasminogen-IGF-I complex, IGF-1 does not compete for binding to IGFBP-3, but instead IGF-1 binds to IGFBP-3-fibrin and binding most likely occurred at the heparin-binding domain. The affinity between the IGFBP-3-fibrin and IGF-1 is similar to the affinity between IGF-1 and type 1 receptor (29). These observations suggest that the fibrin-immobilized IGFBP-3 complex may play a role in concentrating IGF-1 at the wound, aiding in tissue repair. This interaction may be detrimental to the cardiovascular system when tissue repair resides at the vascular level. Also, fibronectin was discovered to bind to both nonglycosylated and glycoslated IGFBP-3. Competitive inhibition of IGFBP-3 was observed in the presence of IGFBP-5 and heparin, whereas IGF-1 and IGFBP-1 had no effect on binding interaction between IGFBP-3 and fibronectin. The binding affinity of the IGFBP-3-fibronectin complex to IGF-1 is similar the affinity of IGF to IGFBP-3 (99).

Although a specific IGFBP-3 cell surface receptor has not been published, there are two putative receptors: one that was cloned via a yeast two-hybrid experiment using the midregion of IGFBP-3 (256) and another termed low-density lipoprotein-related protein-1 (LRP-1)/α2M receptor (117, 146). The former has yet to be validated and the latter has been supported with data from a single laboratory; exact receptor structure and signaling mechanisms are yet to be elucidated. IGFBP-3 bound to the surface of human breast cancer cells through putative high-affinity IGFBP-3 membrane receptors (256). Fragments containing the midregion of IGFBP-3 were able to inhibit radiolabeled IGFBP-3 binding, suggesting that the midregion interacts with the breast cancer cell surface receptor. High affinity binding sites were putatively identified in estrogen receptor (ER)-negative (Hs578T) and ER-positive (MCF-7) human breast cancer cells human. Testing all six IGFBPs indicated only IGFBP-5 was a weak competitor, suggesting IGFBP-3 binding to breast cancer cell receptor is specific and not due to nonspecific glycosaminoglycan interactions (256). The interaction between IGFBP-3 and these receptors suggested that IGFBP-3 may have IGF-1 independent actions on cell growth.

Presumed IGFBP-3 binding to a cell surface receptor induced a rapid and transient increase in intracellular free calcium concentrations in breast carcinoma cells (MCF-7) (201). The increase in calcium concentrations were mediated through a pertussis toxin-sensitive pathway, suggesting its receptor may be coupled to a G_i protein to regulate intracellular signaling. Calcium concentrations increased when IGFBP-3 was bound to heparin or IGF-1, suggesting that receptor binding occurs in the least conserved region (201). Although intracellular localization of IGFBP-3 was not observed in this study, diffuse cellular surface and cytoplasmic staining of IGFBP-3 has been visualized in keratinocytes (248), demonstrating that IGFBP-3 is an intracellular molecule. Still, evidence for IGFBP-3 cell surface receptors and coupled intracellular signaling pathways would be strengthened by confirmation from independent laboratories.

IGFBP-3 was shown to bind to transforming growth factor (TGF)-β type V receptor and inhibited cell growth independent of IGF (117, 146). Surprisingly, matrix-assisted laser desorption/ionization (MALDI) analysis revealed that the TGF-β type V receptor is identical to the low-density lipoprotein-related protein-1 (LRP-1)/α2M receptor (118). An LRP-1 antagonist, receptor-associated protein, inhibited IGFBP-3 binding to ^{125}I-labeled TGF-β1 and ^{125}I-labeled IGFBP-3 binding TGF-β type V receptor. Furthermore, ^{125}I-labeled IGFBP-3 affinity labeled TGF-β type V receptor could be immunoprecipated with antibodies specific to LRP-1 and TGF-β type V receptor, providing additional evidence that the TGF-β type V and LRP-1 receptors are identical (118). Serum IGFBP-3 levels were upregulated in mice with impaired LRP-1 function (Fut8$^{-/-}$ mice), suggesting that LRP-1 is necessary for IGFBP-3 internalization and impaired LRP-1 function results in increased serum IGFBP-3 levels (151). LRP-deficient cells also had reduced IGFBP-3 internalization compared with LRP-expressing mouse embryonic fibroblasts. Treatment with receptor-associated protein inhibited IGFBP-3 internalization in mouse embryonic fibroblasts, indicating that IGFBP-3 is a ligand of LRP-1, and LRP-1 is crucial for internalization (151). Whether the LRP-1 receptor is the primary IGFBP-3 receptor and if this receptor is identical to the breast cancer cell receptor remains unknown.

Yeast two-hybrid analyses indicated that IGFBP-3 binds to latent TGF-β binding protein-1 (LTBP-1), a component of the latent TGF-β complex (100). LTBP-1 is a part of the structural component of the ECM and is a 160- to 240-glycoprotein containing 8-cysteine and calcium binding EGF-like repeats (166). LTBP-1 is involved in sequestration of latent TGF-β in the ECM and delivery of TGF-β to the plasma membrane (166). Binding occurs when IGFBP-3 is bound to IGF-1; the affinity between IGFBP-3 and recombinant LTBP-1 is estimated to be equal or greater than the affinity of IGFBP-3 for fibronectin (99). Of great importance, IGFBP-3 signaling appears to involve an active TGF-β signaling, where IGFBP-3 stimulates phosphorylation of Smad2 and Smad3 to inhibit breast cancer cell proliferation (65, 66). Although in vitro studies indicate that IGFBP-3 and latent TGF-β complex interact via LTBP-1, in vivo studies are warranted to determine the whether LTBP-1 is functionally important in mediating IGFBP-3 binding to plasma membranes (100) and intracellular signaling.

IGFBP-3 possess basic COOH-terminal nuclear localization signals (195), allowing IGFBP-3 to exert intracellular actions. Transferrin associates with IGFBP-3 in the COOH terminus (149, 244); the caveolin-scaffolding domain consensus sequence also resides near the COOH terminal end and IGFBP-3 bound to caveolin-1 on breast cancer cells (27). These facts lead to the discovery that caveolin-1 mediates IGFBP-3 inter-

nalization via a caveolin-scaffolding docking sequence (149). Caveolae are glycolipid rafts that are involved in endocytosis and signal transduction (226), and caveolin-1 has been implicated to be a principle structural scaffold that organizes intracellular trafficking and cytoplasmic signal transduction (85, 205), providing a mechanism for IGFBP-3 internalization and signaling.

Coimmunoprecipitation assays demonstrated that IGFBP-3 binds to transferrin, which in turn binds to its receptor to form a ternary complex. Cotreatment of anti-transferrin receptor antibody and cholesterol depletion agents completely abolished endogenous and exogenous IGFBP-3 uptake. This suppression also inhibited IGFBP-3-mediated apoptosis in prostate cancer cells to show functional importance of endocytic internalization. Caveolae, transferrin, and the transferrin receptor function together to collectively mediate endocytosis of IGFBP-3 (149). Whereas transferrin is required for full-length IGFBP-3, it is not required for IGFBP-3 fragments lacking the transferrin-binding domain in breast cancer cells (27). A serine phosphorylation domain peptide (SPD) that lacks the region that interacts with transferrin is still able to induce C2-ceramide cell death in Hs578T cells, indicating that transferrin is not essential for IGFBP-3 action. A related study reported that cellular uptake of IGFBP-3 is mediated by metal ion-stimulation of the MBD (223). Full-length IGFBP-3 was shown to bind nickel, iron, and zinc via the MDB; the MDB was reported to have physical interaction with caveolin-1 and transferrin receptor. Thus the MDB may be involved in intracellular translocation of IGFBP-3 (223). IGFBP-3 binding to nickel or iron-charged resin was inhibited in the presence of IGF in vitro; however, whether MDB-mediated internalization occurs in an in vivo model remains to be determined.

Autocrine motility factor/phosphoglucose isomerase (AMF/PGI) was identified as a binding partner for IGFBP-3 in solubilized T47D and MCF-7 human breast cancer cell membranes (176). AMF/PGI is endocytosed in tumor cells and is utilized as a cell-penetrating peptide to deliver toxins to the intracellular space (136), similar to cell-penetrating peptides derived from IGFBP-3 and IGFBP-5 (90). IGFBP-3 antagonized its induction of migration of these breast cancer cells. Whether a classical receptor will be discovered and characterized for IGFBP-3 or whether the "receptors" described in the current paper are part of larger protein complexes in an endocytic pathway remains an unanswered question.

Nuclear Binding Partners

IGFBP-3 had previously been shown to translocate into the nucleus in opossum kidney cells (156); recombinant IGFBP-3 has been visualized in human breast cancer cells (T47D) (213); endogenous nuclear IGFBP-3 has also been detected in human lung cancer cells (127) and during cell division in keratinocytes (248). IGFBP-3 also has been demonstrated to localize to the nuclei of myoblasts through MBD interaction, where IGFBP-3 bound to RNA polymerase II binding subunit 3 (Rpb3) (188). IGFBP-3 interaction with Rpb3 is dependent on a functional NLS as a NLS mutant failed to associate with Rpb3. Since Rpb3 regulates gene transcription and gene expression through recruitment of the polymerase complex to specific transcription factors, interaction between IGFBP-3 and Rpb3 could provide a functional role for IGFBP-3 in modulation of gene transcription.

Initial findings in human breast cancer cells (T47D) suggest that NLS of the COOH terminal region is involved in nuclear import of recombinant IGFBP-3 (213). Using fluorescently labeled IGFBP-3, IGFBP-3 was rapidly taken up during rapid cell division. In a follow-up study, wild-type IGFBP-3 underwent translocation, but not a NLS mutant form, through a NLS-dependent pathway into an intact nuclear envelope. When the nuclear envelope was permeabilized, wild-type IGFBP-3, but not the mutant form, accumulated in the nucleus, suggesting that the NLS was involved in binding IGFPB-3 to the nuclear components. IGFBP-3 was recognized by importin-β and α/β-heterodimer, suggesting that IGFBP-3 nuclear importation is dependent on its NLS and importin-β nuclear transport factor (210). When the plasma membrane is compromised (i.e., detergents), nuclear transport of IGFBP-3 occurs in almost all cells (210).

IGFBP-3 interacts with nuclear receptors: retinoid X receptor (RXR)-α (159), RAR (212), and Nur77 (148). Binding to RXRα was identified using multiple methods, including a yeast two-hybrid screen (159). Evidence of IGFBP-3-RXRα functional interaction was further demonstrated by abrogation of IGFBP-3-induced apoptosis in RXRα-knockout cells, indicating that RXR is required for IGFBP-3-induced apoptosis. Combination treatment of IGFBP-3 and RXR ligands synergistically induced apoptosis in prostate cancer cells (161).

IGFBP-3 sequestered RAR and blocked the formation of RAR:RXR heterodimers (212). The presence of IGFBP-3 substantially reduced the number of binding sites for all-trans retinoic acid (atRA), a specific ligand for RARα, decreasing atRA binding to RAR:RXR heterodimers. To show interaction in a different way, IGFBP-3 depletion from an in vitro system increased sensitivity of RA-resistant breast cancer cells to the growth inhibitory effects of atRA (212). Residues within the NH_2 terminal and motifs within the COOH terminal are essential for interaction with RXRα and RARα and subsequent modulation of RAR/RXR signaling (209).

IGFBP-3 induces RXRα-Nurr77 translocation from the nucleus to mitochondria (150). IGFBP-3 treatment was observed to induce a rapid appearance of extra nuclear GFP-Nur77 in $RXR\alpha^{+/+}$ cells, but not in $RXR\alpha^{-/-}$ cells, as observed with confocal microscopy. Mitochondrial isolation from cells transfected with IGFBP-3 showed a threefold increase in mitochondrial RXRα, whereas this response was absent in cells transfected with control expression vector. IGFBP-3 trafficking to organelles other than the nucleus has yet to be described. Figure 2 summarizes IGFBP-3 structure and intracellular trafficking. Figure 3 outlines IGFBP-3 binding partners in relation to its potential function.

REGULATION OF IGFBP-3 TRANSCRIPTION, AVAILABILITY, AND ACTION

IGFBP-3 affinity to IGF controls bioavailability and action of IGF (Fig. 4). For example, IGFBP-3 is able to sequester IGF from its receptor because IGFBP-3 has greater affinity for IGF than it does for its receptor, thus reducing IGF bioavailability and action. However, proteolysis and fragmentation of IGFBP-3 (44, 141, 241) and attachment of IGFBP-3 to proteins on the extracellular matrix (172) reduces its affinity to IGF.

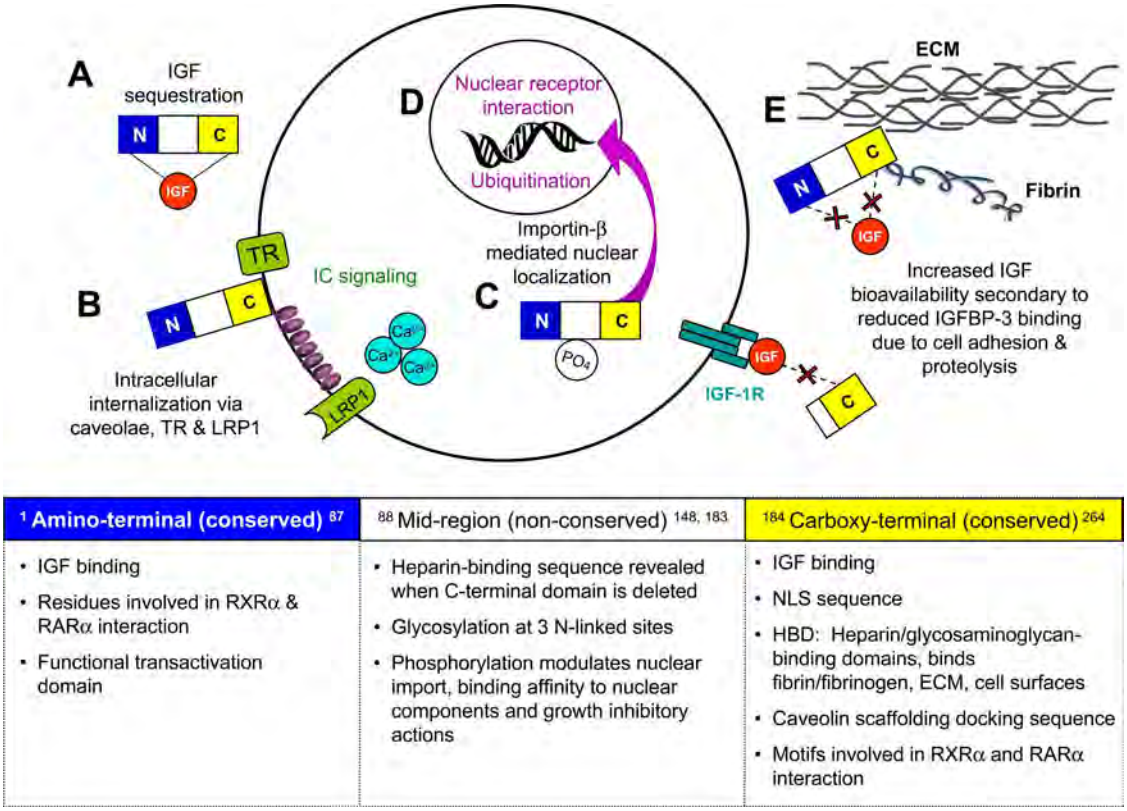

Fig. 2. IGFBP-3 and cellular interaction. *A*: IGF binds at the NH_2 and COOH terminus; although this figure depicts IGFBP-3 as a linear structure, its proposed tertiary structure has been described as a horseshoe, allowing an IGF molecule to bind at both the NH_2 and COOH terminus simultaneously. The IGFBP-3:IGF-1 affinity is greater than IGF-1:IGF-R affinity, resulting in IGF sequestration. *B*: caveolin-1, transferrin, and the transferrin receptor (TR) function together to mediate endocytosis of IGFBP-3 and may be involved in intracellular trafficking and cytoplasmic signaling transduction. IGFBP-3 also may be internalized via lipoprotein-related protein (LRP)-1. Whether LRP-1 and the breast cancer cell receptor are identical or which terminus interacts with LRP-1 is unknown. IGFBP-3 may initiate a rapid increase in calcium concentrations and regulate intracellular signaling. *C*: phosphorylation of IGFBP-3 may increase its nuclear import. Nuclear localization of IGFBP-3 is mediated by nuclear transport protein importin-β, allowing interaction with nuclear transcription factors. DNA-PK can phosphorylate nuclear IGFBP-3 to modulate DNA repair/replication and apoptosis. *D*: ubiquitination of IGFBP-3 occurs in the nucleus, and one of its 19 lysine residues probably serves as an anchor for polyubiquitination. *E*: interaction between the COOH terminal of IGFBP-3 and proteins attached to the extracellular matric (ECM) [i.e., latent tranforming growth factor-β binding protein (LTBP-1)] or type 1α collagen affects cell adhesion and migration. Binding to ECM or fibrin decreases the affinity of IGFBP-3 to IGF-1, increasing IGF bioavailability. Proteolyzed IGFBP-3:IGF-1 affinity is less than IGF-1-R:IGF-1 affinity, preserving IGF-IR sensitivity secondary to IGF-1 binding. Superscript numbers in IGFBP-3 molecule represent residues within each domain, with varying residues in the nonconserved domain depending on the presence or absence of the heparin binding domain. RXR, retinoid x receptor; RARα, retinoic acid receptor.

The result is enhanced IGF bioavailibility and augmented IGF action at its receptor. In addition to proteolysis, IGFBP-3 activity can be affected by other posttranslational modifications such as phosphorylation, methylation, glycosylation, and ubiquitination. IGFBP-3 levels are also affected by the rate of its transcription. IGFBP-3 gene transcription is affected by numerous agents, such as retinoic acid (RA), estrogen, p53, TGF-β, tumor necrosis factor (TNF-)-α, vitamin D, sodium butyrate (NaB), and follicle stimulating hormone (FSH) (187). This section will review different ways in which IGFBP-3 binding affinity, trafficking, and activity can be modulated.

Regulation of Gene Transcription

Vitamin D. 1,25-Dihydroxyvitamin D3 (vitamin D) and its analogs have been shown to decrease cell proliferation in prostate carcinoma cells in vitro and in rat prostate in vivo; this response is associated with increased IGFBP-3 expression (181). This observation is well documented in LNCaP cells, an androgen-dependent cell line (22, 170). An approximate threefold induction of IGFBP-3 mRNA expression and protein secretion in LNCaP cells has been reported after a 24-h exposure to vitamin D (170). Moreover, 4 days of exposure to vitamin D plus sense oligodeoxynucleotides (ODN) inhibited cell growth in LNCaP cells, whereas this response was abrogated in antisense ODN and vitamin D treatment (22). Treatment induced an increase in IGFBP-3 mRNA as soon as 6 h posttreatment with a maximal response observed 12 h posttreatment (22).

Vitamin D analogues have been studied as anti-cancer therapies because they do not exert hypercalcemic effects like their naturally bioactive counterparts. A variety of homologues have been used to induce IGFBP-3 expression and/or secretion from a variety of cell lines [i.e., human osteosarcoma cells, PC-3 (121), Hs578T, and MCF-7 (46)]. The anti-proliferative actions of vitamin D are mediated through vitamin D receptor (VDR), a classic nuclear receptor that is usually composed of two direct repeats of 6 bases, separated by a 3-nucleotide spacer (CDR3 motif) (139). A 15-bp sequence containing 2 hexameric core sites were identified as a potential IGFBP-3 vitamin D response element (VDRE) (192). 1,25 OH_2D_3 re-

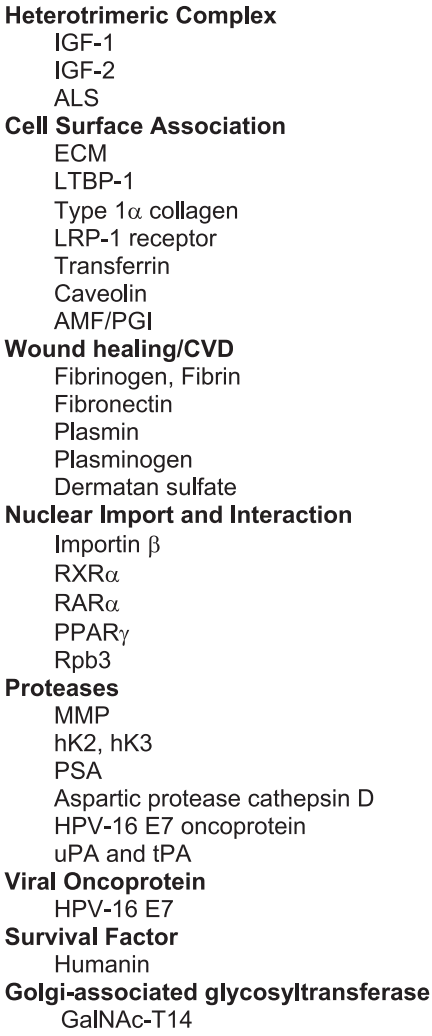

Fig. 3. IGFBP-3 binding partners, organized by potential function. See text for more definitions.

cruited the VDR/RXR heterodimer to the VDR site in the IGFBP-3 promoter; mutations of this element abrogated the proper hexameric sequence (192). It has been suggested that vitamin D interacts with RXR since both VDR and RAR act through this common nuclear receptor. Normal action of a specific RAR antagonist AGN193109 antagonized retinoids but failed to antagonize vitamin D in human papillomavirus-immortalized cervical epithelial cells, which suggests vitamin D acts through VDR and not retinoid signaling molecules (4). Thus vitamin D may involve signaling through nuclear receptors VDR/RXR but not RAR/RXR to increase IGFBP-3 gene expression.

Steroid receptor coactivator 3 (SRC-3/AIB1/ACTR/NCoA-3) is a transcriptional coactivator for nuclear receptors including VDR (158). Rapid serum IGF-I degradation in SRC-3$^{-/-}$ mice was associated with a drastic reduction of serum IGFBP-3 levels that cannot be restored by transgenic IGF-I compensation. In addition, disruption of SRC-3 in mice decreased the expression of IGFBP-3 and several other known VDR target genes. SRC-3-deficient cells were unable to support vitamin D-induced IGFBP-3 promoter activity, and IGFBP-3 mRNA was reduced in SRC-3$^{-/-}$ mice. SRC-3 maintained circulating IGF-I by controlling IGFBP-3 expression (158).

p53. The DNA elemental sequence of IGFBP-3 to which p53 interacts in vitro was identified as a consensus binding site containing two copies of a 10-bp motif separated by 0–13 bp (62). Alterations of this motif lead to a loss of p53 affinity. In addition, p53 mutants failed to bind to the consensus dimmer; these mutants represented altered "hot spots" often present in human cancer that results in a loss of tumor suppression. Two p53-responsive elements were identified in the first and second introns of the IGFBP-3 gene, and highly purified wild-type p53 bound to the consensus element, whereas the mutant p53 failed to bind (25). These investigators used a differential cloning approach to demonstrate that the IGFBP-3 gene coding is regulated by p53 binding. Activation of this p53 transgene increased in IGFBP-3 mRNA expression and protein in media conditioned by colon carcinoma cells, cells that have an inducible wild-type p53 transgene and undergo apoptosis upon p53 induction (25). These results are consistent with their previous observation that activation of osteosarcoma cells containing temperature-sensitive p53 element Saos-2-D4H lead to increased IGFBP-3 mRNA expression (24).

Functional p53 domains dictate target gene specificity such that different isoforms differentially regulated target gene expression. Activation domain 2 (AD2) was required for p53-dependent apoptosis (263) and the COOH-terminal basic domain (BD) is a regulatory domain where deletion increases p53-specific binding activity in vitro (106). Interestingly, naturally occurring isoforms of p53 with truncated COOH-terminals induced IGFBP-3 expression, whereas full-length forms could not induce expression. Furthermore, histone deacetylase (HDAC) activity restores the ability of exogenous full-length p53 to induce IGFBP-3. Simultaneous p53 DNA stabilization and inhibition of HDAC restored the ability of endogenous full-length p53 to induce IGFBP-3 (106).

Since p53 modulates IGFBP-3 gene transcription, factors that affect p53 are also capable of regulating IGFBP-3 availability. For example, total body irradiation (and resultant DNA damage) induced p53 in the thymus and small intestine of p53$^{+/+}$ female mice, resulting in increased IGFBP-3 mRNA as early as 6 h postirradiation (95). A human lung carcinoma cell line transfected with a plasmid containing the gene E6 contains less p53 versus the same lung carcinoma cell transfected with an empty plasmid. After exposure to etoposide, a chemotherapeutic agent, both cell types increased IGFBP-3 secretion into conditioned media (95). Treatment with adriamycin, a genotoxic drug, increased IGFBP-3 secretion in p53$^{-/-}$ PC-3 cells showing that IGFBP-3 can also be induced independent of p53-mediated gene transcription (95).

Of note, p53 activity (10) and IGFBP-3 mRNA was greatly increased during cellular senescence in human diploid fibroblasts (91) and human prostate epithelial cells (214). Since IGFBP-3 gene activity was activated by p53, this suggested that p53 activity during cellular senescence may be required for induction of IGFBP-3. Increases in p53 were accompanied with consistent IGFBP-3 mRNA levels in all conditions, indicating that p53 activity does not affect IGFBP-3 mRNA. Thus increased p53 activity is associated with increased IGFBP-3 mRNA; however, p53 did not contribute to IGFBP-3 gene overexpression in this system (165).

Hypoxia. Hypoxia can induce IGFBP-3 mRNA through p53-independent (67, 95) and -dependent mechanisms (39). Induction of IGFBP-3 mRNA was observed in response to

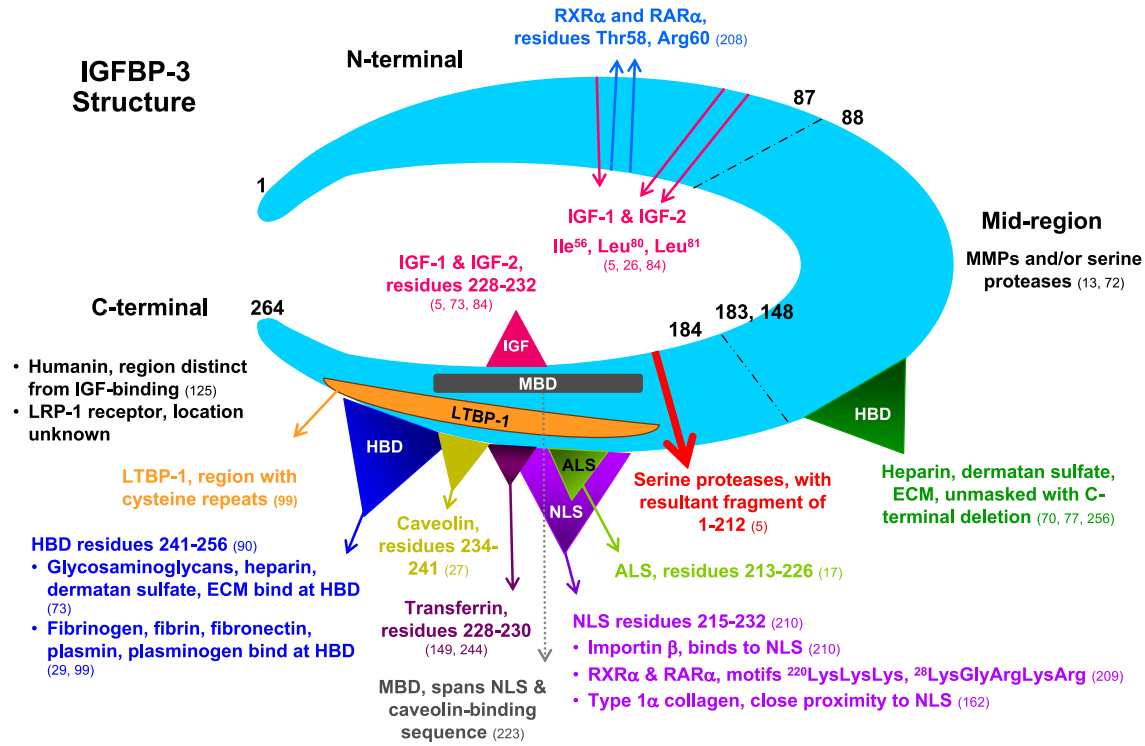

Fig. 4. IGFBP-3 binding partners. Dashed line separates IGFBP-3 into 3 domains; numbers represent residues at the end of each domain. Binding partners are depicted within the region/sequence involved in molecular interactions.

hypoxia in wild-type embryonic stem cells (67). Increasing dosages and hypoxic exposure time both increased IGFBP-3 mRNA in a dose-dependent manner in $p53^{+/+}$ lung carcinoma cells (95). Still, when these methods were repeated using $p53^{-/-}$ PC-3 cells, similar results were observed, suggesting that hypoxia can modulate IGFBP-3 mRNA expression independent of p53. In fact, many cancer cell lines had IGFBP-3 mRNA induction by hypoxia regardless of their p53 load/status (95).

Androgen. Androgen actions on IGFBP-3 transcription are mediated by the androgen receptor (AR) (87), in which the AR binds as a homodimer to the androgen response elements (ARE) in the distal region of the IGFBP-3 promoter (193, 239). Some investigators have shown that androgens have inhibitory effects on IGFBP-3 expression in LNCaP cells, an androgen-dependent human prostate cancer cell line (9, 92, 137). Alternatively, androgen treatment has been shown to stimulate IGFBP-3 expression in the same cell line (170). A possible explanation for the discrepancy is the biphasic response of androgen treatment on LNCaP cells, where low doses of androgen stimulated growth, whereas high concentrations resulted in growth inhibition (147).

Methylation

Abberant DNA methylation is the most common molecular abnormality in cancer (126). It involves epigenetic modification at the CpG island (cytosine-phosphate-guanine site) in the promoter region, to cause chromatin condensation and gene silencing (245). Hypermethylation of the CpG islands may result in transcriptional silencing by physically blocking the binding of the transcription proteins to the promoter. Alternatively, methylated DNA may be bound by proteins referred to as methyl-CpG-binding domain proteins, which in turn recruit additional proteins to the locus (i.e., histone deacetylases, chromatin remodeling proteins) that are capable of modifying histones and silencing genes. Since IGFBP-3 is a tumor suppressor gene, silencing the IGFBP-3 gene would presumably increase tumor growth.

Poor prognosis in stage I non-small cell lung cancer was associated with IGFBP-3 methylation (37, 38). This relationship was illustrated in another study that showed aberrant DNA methylation caused a reduction in IGFBP-3 expression by 75% in hepatocellular carcinomas (104). Similarly, hepatitis B virus X-protein (HBx), a transcriptional transactivator of viral and cellular promoters, repressed IGFBP-3 by promoting HBx/HDAC1 complex formation (218). Hepatitis B virus (HBV), which increases the risk of hepatocellular carcinoma, encodes oncogenic HBx. HBx recruited HDAC1 and deacetylated specificity protein (Sp)-1 in a p53-independent fashion, resulting in reduced Sp1 binding onto targeted DNA and suppression of IGFBP-3 (218). Methylation plays a critical role in tumorgenesis through gene silencing and chromatin remodeling.

Growth Inhibitory Agents

Anti-estrogen. Pure anti-estrogen, ICI-182780, and Tamoxifen, a partial ER antagonist, increased IGFBP-3 secretion from MCF-7 cells, whereas cotreatment of anti-estrogens with estradiol reversed this effect (194). The capacity of ICI-182780 to stimulate IGFBP-3 gene transcription was 10 times that of Tamoxifen (120). Nuclear run-off assays showed that IGFBP-3 gene transcription was increased by ICI-182780 and decreased by estradiol in MCF-7 cells (122). IGFBP-3 mRNA was three to four times greater in ICI-182780 treated MCF-7 cells versus controls and more than 20 times greater compared with estradiol-treated cells. To demonstrate that IGFBP-3 is integral in

mediating anti-prolific actions, recombinant IGFBP-3 was used to inhibit basal and estradiol-stimulated cell proliferation, and IGFBP-3 antisense ODN abrogated the growth inhibitory effects of anti-estrogen (122).

TGF-β. Anti-estrogen treatment stimulated TGF-β secretion 8- to 27-fold in MCF-7 cells, resulting in attenuated ER-negative human breast cancer cell growth. This effect was reversed with anti-TGF-β antibodies, suggesting that TGF-β is hormonally regulated and may have autocrine and paracrine functions in breast cancer cells (135).

An increase in both IGFBP-3 mRNA expression and IGFBP-3 secretion was observed within 8-h and 2-days post-TGF-β2 treatment of ER-negative human breast cancer cells (Hs578T), respectively; 4 days later cell growth was suppressed (185). Gucev et al. (97) repeated this experiment in a different human breast carcinoma cell line that is IGF-1 negative (MDA-MB-231). Their data illustrated that 3 days of TGF-β2 treatment resulted in two- to threefold increase in IGFBP-3 protein levels in conditioned media. A 5-day treatment of exogenous recombinant human nonglycosylated IGFBP-3 reduced cell survival in a dose-dependent fashion. Since this cell line is devoid of IGF-1, results suggested that IGFBP-3 inhibits cell proliferation in an IGF-1 independent manner.

TGF-β was observed to be a potent stimulator of IGFBP-3 production in fibroblasts (168). In PC-3 cells, TGF-β treatment induced a large elevation of IGFBP-3, which lead to apoptosis (197). IGFBP-3 sense and TGF-β1 induced apoptosis, whereas an IGFBP-3 antisense oligomer blocked TGF-β1-induced apoptosis in PC-3 cells, suggesting that IGFBP-3 is a crucial intermediary step in mediating apoptosis. Apoptosis was attenuated when neutralizing antibodies of IGFBP-3 blocked TGF-β induction of apoptosis, providing additional evidence that increased IGFBP-3 expression is critically involved in TGF-β-mediated programmed cell death.

IL-1. Treatment of interleukin (IL)-1β caused a time- and dose-dependent induction of IGFBP-3 mRNA in Leydig cells. This treatment caused an increase in IGFBP-3 transcription, contributing to the inhibitory effects of IL-1β on cell steroidogenesis (243). IL-1β treatment also caused a dose-dependent increase in IGFBP-3 in conditioned media; IL-1β treatment increased IGFBP-3 transcription rate and did not change the stability of IGFBP-3 mRNA.

Whereas IL-1β is produced by activated macrophages and is readily involved in pro-inflammation (59), IL-1α is constitutively expressed in testes, localized to Sertoli cells, and is involved in differentiation of Leydig cells in rats (128). IL-1α-stimulated Leydig cells resulted in increased IGFBP-3 secretion (45). However, when Leydig cells were incubated with both GH and IL-1α, intact IGFBP-3 levels decreased in culture media. Furthermore, cell culture media catalyzed the degradation of IGFBP-3 with simultaneous appearance of fragments, suggesting Leydig cells produce an IGFBP-3 protease (45).

IL-6. Prominent IL-6 production is commonly observed in systemic juvenile idiopathic arthritis and this disease is accompanied by stunted growth and decreased IGFBP-3 and IGF-1 levels (56). While treatment with IL-6 increased biosynthesis of IGFBP-3 protein and total mRNA in a dose-dependent manner in cocultures of hepatocytes and Kupffer cells (152), rhIL-6 treatment in CB6F1 mice resulted in a significant decrease in IGFBP-3 levels compared with saline-treated mice (56). Elevated pro-inflammatory cytokine IL-6 decreased IGFBP-3 levels and caused a decreased association of IGF-1 in the 150-kDa complex. Thus growth impairment may be attributed to IGFBP-3 reduction and shortened half-life of IGF-1 as decreased IGF-1 levels appeared to be secondary to increased clearance.

Chronic overexpression of pro-inflammatory cytokine IL-6 in transgenic mice resulted in a 50% reduction in circulating IGFBP-3 levels and a marked decrease in growth rate so that adult transgenic mice are 50–70% the size of control littermates (55). The decrease in IGFBP-3 was in part attributed to increased proteolysis as intact IGFBP-3 protein was decreased in transgenic mice. Whereas the mechanism explaining the IL-6-stimulated reduction in IGFBP-3 levels is not clear, IL-6 may directly affect IGFBP-3 production or the effects may be secondary to IGFBP-3 proteolysis.

TNF-α. Pro-inflammatory cytokine TNF-α has been reported to increase IGFBP-3 secretion and mRNA expression in MCF-7, resulting in inhibition of cell proliferation (206). TNF-α abrogated the inhibitory effects of estradiol on IGFBP-3, resulting in IGFBP-3 accumulation. This response has also been observed in cultured rat aortic vascular smooth muscle cells (VSMC), which normally secrete only trace amounts in IGFBP-3 (8). In the absence of TNF-α stimulation, IGFBP-3 could not be detected in conditioned media or with Northern blots. Treatment with increasing dosages of TNF-α over a 24-h period increased both IGFBP-3 secretion and mRNA expression with no change in other IGFBPs. Blocking TNF-α induction with cycloheximide and actinomycin D inhibited induction of mRNA expression and protein secretion of IGFBP-3, providing further evidence that TNF-α regulates IGFBP-3 (8).

Retinoic acid. As an active derivative of vitamin A, retinoic acid (RA) regulates the growth of a variety of cell lines, including human breast cancer cells (2, 97, 169, 215), osteoblast cells (262), and cervical epithelial cells (109). RA antagonizes cellular proliferation of human breast carcinoma cells presumably by increasing secretion of anti-proliferative IGFBP-3 in vitro (74). However, others observed secretion of IGFBP-3 from this cell line, perhaps only in the absence of estradiol stimulation (120, 169). atRA, a FDA-regulated drug, caused a threefold increase in IGFBP-3 expression in a human breast cancer cell line, indicating de novo synthesis of IGFBP-3 (2).

In a separate study, lower atRA concentrations stimulated a twofold increase in IGFBP-3 protein secretion into conditioned media (169). Dose-dependent increases in IGFBP-3 mRNA expression with atRA treatment was observed in MCF-7 cells (215). Increasing treatment durations resulted in a dose-dependent increase in IGFBP-3 mRNA expression with maximal expression achieved after 48 h. RARβ expression was induced with atRA stimulation through an RARα-dependent pathway in MCF-7 (215) cells and human bronchial epithelial cells (103).

In a human breast carcinoma cell line, RA resulted in a two- and threefold increase in IGFBP-3 mRNA and protein in conditioned media, respectively (97). To show that IGFBP-3 was essential for the anti-proliferative effects of RA, cells were treated with a combination of antisense IGFBP-3 ODN and RA. This treatment (but not sense ODN) failed to increase IGFBP-3 protein in conditioned media by 80%, indicating

IGFBP-3 is directly involved in mediating inhibition of cell proliferation (97). Similar responses to RA have been documented in cervical epithelial cells, human osteoblast cells (262), and human prostate adenocarcinoma cells (123).

Sodium butyrate. As a histone deacetylase inhibitor and 4-carbon fatty acid, sodium butyrate (NaB) exerts different morphological and biochemical effects in cell culture through regulation of nuclear enzymes (191) to ultimately affect gene expression and cell growth (140). For example, it inhibited hyperacetylation of histones H3 and H4 (202) resulting in increased transcriptional activity in HeLa cells (60). Other effects include inhibition of protein methylation (20), reduced RNA polymerase molecules and activity (164), increased ADP-ribose polymerase activity (191), and more recently discovered are its apoptotic effects on prostate cancer cells (32). NaB transcriptionally upregulated IGFBP-3 mRNA and protein levels in PC-3 and LNCaP cells in both dose- and time-dependent manners, with maximal induction after 2-h of treatment (234). NaB involves two distinct regions in the IGFBP-3 promoter in PC-3 and LNCaP prostate cancer cells. PC-3 cells are p53 null, implying NaB acts independently of p53 (234). It has been proposed that NaB increases IGFBP-3 expression in breast cancer cells by activating the IGFBP-3 promoter through a Sp1/Sp3 multiprotein complex (242).

Growth Stimulatory Agents

Growth hormone and IGF-1. In systemic models, IGF-1 and IGFBP-3 are correlated with GH secretion, as patients with GH deficiency (GHD) have reduced serum concentrations for all three proteins, and patients with acromegaly have increased levels (105, 107). Systemic GH administration corrects serum IGF-1 and IGFBP-3 concentrations in GHD (19), whereas IGF-1 treatment yields inconsistent effects. Chronic IGF-1 treatment of GH insensitivity increased serum IGFBP-3 in patients (130), whereas additional studies failed to show increases in IGFBP-3 concentrations (98, 144).

In a different systemic model, hypophysectomized rats have been used as a model to evaluate the relationship among IGFBP-3, GH, and IGF-1. Recombinant human GH (41, 259) and IGF-1 (41, 89) infusion induced a sevenfold increase in IGFBP-3 in hypophysectomized rats. In GH-deficient dwarf rats, GH or IGF-1 infusion induced IGFBP-3 mRNA levels in skin, whereas only GH infusion induced message levels in muscle and liver (153). Since changes in IGFBP-3 and IGF-1 occur in parallel, this suggests GH mediates IGFBP-3 induction in part by IGF-1 (41).

Furthermore, local GH and IGF-1 treatment caused a threefold induction of IGFBP-3 protein in media conditioned by human hepatocarcinoma cells in a dose-dependent manner (96). IGFBP-3 mRNA was increased as early as 6 h post-GH treatment, whereas mRNA levels increased 24 h post-IGF-1 treatment. Northern blots also showed that GH had an earlier transcriptional effect on IGFBP-3 mRNA compared with IGF-1. These observations suggested GH may act independently of IGF-1 when augmenting IGFBP-3 mRNA levels. To show that GH or IGF-1 did not affect IGFBP-3 through alteration of mRNA stability, the half-life of IGFBP-3 was measured to be 14–18 h, and this was unchanged by either treatment. Collectively, these results indicated that IGF-1 and GH are true regulators of IGFBP-3 transcription (96).

Epidermal growth factor. Marked reduction of IGFBP-3 levels were observed post-EGF treatment in ECE16–1 cells (human papillomavirus-immortalized cervical epithelial cells) (7). Also, addition of mitogenic EGF to basal human keratinocyte cells (HaCaT) resulted in a 20-fold reduction in IGFBP-3 mRNA (249). Thus epidermal mitogens such as EGF may increase local IGF-1 availability by inhibiting IGFBP-3 synthesis.

Posttranslational Modifications

Proteolysis. Metabolic demands appear to be a major determinant of IGFBP-3 proteolytic activity. For example, pregnancy, NIDDM, and recuperation after surgery or illness results in increased proteolytic activity. Significant IGFBP-3 proteases have been described in extravascular fluids, such as interstitial (252), synovial (68), follicular (50), and peritoneal fluids (119).

Increased IGFBP-3 proteolytic activity has been described in serum from pregnant women (5, 116). Analyses revealed that IGFBP-3 detected in serum obtained during the second month of pregnancy had reduced affinity for IGF-1 and IGF-2 (116). In addition, immunoreactivity to IGFBP-3 was detected at 30 kDa, while 41.5- and 38.5-kDa bands were not detected. These findings could most likely attributed to protease activity (116). Proteolytic modification of IGFBP-3 resulted in cleavage of the midregion, presumably by MMPs (metalloproteases) and serine proteases as determined in vitro (14, 78).

In addition, Western blotting of human pregnancy serum obtained from healthy volunteers during the third trimester revealed a single 30-kDa band and occasional bands at 16 and 20 kDa, whereas nonpregnancy serum contained IGFBP-3 immunoreactivity at 40–43 kDa and 30 kDa (5). Mass spectrometry and amino acid sequence analysis identified the 30-kDa proteolytic fragment obtained from pregnancy serum by the NH_2- and COOH terminus. The fragment corresponded to residues 1–212 of intact IGFBP-3, indicating cleavage occurred in the COOH terminal. The proteolytic fragment was reported to have an 11-fold decreased affinity for IGF-1 and 4-fold decreased affinity for IGF compared with intact IGFBP-3. Even if proteolytic fragments of IGFBP-3 have reduced affinity for IGF, these fragments may still inhibit both IGF and insulin-induced cell growth (141, 142).

The increased proteolytic activity during pregnancy is attributed to the intense cell growth and metabolism. Similarly, increased IGFBP-3 proteolytic activity was observed in serum from NIDDM patients (12). Proteolytic activity was inhibited by serine protease inhibitors, and the authors believed that the increased proteolytic activity in serum from NIDDM (and pregnant) patients resulted from reduced levels of protease inhibitors and not increased proteolytic activity per se (12, 14). Insulin regulated IGFBP-3 proteolytic activity in NIDDM serum, and proteolysis increases IGF bioavailability (14). In addition, IGFBP-3 proteolysis may counteract the resultant catabolic postsurgery state (52). It is believed that increased proteolysis is a compensatory mechanism that increases IGF bioavailability (53).

Prostate-specific antigen (PSA) and human prostatic kallikreins (hK2, hK3) are also capable of inactivating IGFBP-3 by proteolytic degradation. PSA is a serine protease found in seminal plasma that cleaves IGFBP-3 resulting in a marked reduction in binding affinity of the fragments to IGF1 but not

IGF-2 (43). hK2 and hK3 are IGFBP-3 proteases, with hK2 being more effective at lower concentrations (198). Heparin remarkably enhances the ability of hK3 (but not hK2) to degrade IGFBP-3, thus a combination of these molecules may influence IGFBP-3 and the proliferation of normal and abnormal prostatic cell (198).

Aspartic protease cathepsin D has been shown to cleave IGFBP-3, whereas depletion of cathepsin D attenuates proteolysis (47). Urokinase-plasminogen activator (uPA), tissue-plasminogen activator (tPA), plasmin, and plasminogen are capable of modulating IGFBP-3. uPA and tPA were used to proteolyze IGFBP-3 (13), and this may increase IGF-1 bioactivity. These plasminogen-related molecules have been used to dissociate IGF from IGFBP-3 in osteosarcoma cultures, whereas dissociation was inhibited with plasminogen activator inhibitor (PAI)-1 and uPA antibodies (31). Whereas IGFBP-3 binds plasminogen at the HBD, the IGFBP-3-plasminogen complex can also binds to IGF-1 with high affinity to decrease IGF-1 bioavailability (30). In addition, the human papillomavirus (HPV-16) E7 oncoprotein also enhanced proteolytic degradation of IGFBP-3 while proteolysis is inhibited by proteasome inhibitors (167). In summary, IGFBP-3 proteolytic degradation may be regulated through metabolic demands and results in modulation of its anti-apoptotic actions and bioavailability of IGF.

Phosphorylation. Phosphorylation is an important posttranslational modification of IGFBP-3 not only because IGFBP-3 is secreted as a phosphoprotein, but phosphorylation is a critical regulator of nuclear import of proteins (211). The phosphoprotein IGFBP-3 is constitutively phosphorylated at casein kinase 2 (CK2) sites (49). IGFBP-3 has two phosphorylation sites contained within consensus sites, Ser^{111} and Ser^{113} (111). Dephosphorylation of these sites by CK2 appear to enhance its binding to ALS and to cellular surfaces (49, 111).

IGFBP-3 can be phosphorylated in vitro by CK and cAMP-dependent protein kinase (PKA) (49). Double-stranded DNA-dependent protein kinase (DNA-PK) can phosphorylate three potential sites in the nonconserved domain of IGFBP-3: Ser^{156}, Ser^{165}, Thr^{170} (134). All three sites are highly conserved among human, bovine, pig, rat, and mouse IGFBP-3 (211). DNA-PK is present in both the cytosol and nucleus (251); however, it mainly acts in the nucleus (211). It is activated by DNA damage and may be involved in DNA strand repair, replication, gene transcription, and recombination of 2′-deoxysio-guanosine (iG) genes (227).

DNA-PK phosphorylation of IGFBP-3 was shown to reduce its affinity for IGF-1 and -2, whereas CK2, PKA, cdc2 (cyclin-dependent protein kinase), and MAPK (mitogen-activated protein kinase) had no affect on ligand binding in a binding assay (211). Interestingly, in vitro IGFBP-3 phosphorylation by DNA-PK increased IGFBP-3 nuclear import resulting in elevated maximal levels of nuclear accumulation, whereas other kinases failed to increase nuclear import. It is possible that DNA-PK acts in the cytosol to enhance IGFBP-3 nuclear import.

Furthermore, inhibiting IGFBP-3 phosphorylation at two of the three putative DNA-PK phosphorylation sites (Ser^{165} and Thr^{170}) in 22RV1 cells with site-directed mutagenesis resulted in apoptosis identical to the wild-type IGFBP-3 (42). However, mutation of Ser^{156} inhibited apoptosis and reduced nuclear accumulation. These results suggest that phosphorylation IGFBP-3 by DNA-PK regulates the growth inhibitory role of IGFBP-3. Taken together, cumulative data suggest that IGFBP-3 phosphorylation can affect nuclear import, its binding affinity to other nuclear components, and growth inhibitory actions.

Glycosylation. Each of the three potential *N*-glycosylation sites in central region of IGFBP-3, Asn^{89}, Asn^{109}, Asn^{172} increases the molecular weight of IGFBP-3 by 4, 4.5, and 0–5 kDa, respectively (71). The absence of *N*-glycosylation at any site does not prevent its secretion from the cell. However, glycosylation may confer IGFBP-3 protease resistance by physically protecting susceptible sites or increasing the rigidity of the protein (72). The interaction of IGFBP-3 with cellular binding elements and IGFs has been speculated to involve negative charges on the targets. Charge reversal of the target significantly reduces binding affinity of the peptides, contributing to alterations in protein-protein, protein-cell, and cell-cell interactions (72). In addition, *N*-acetylgalactosaminyltransferase 14 (GalNAc-T14) was identified as an IGFBP-3 binding partner via yeast two-hybrid screens and glutathione-*S*-transferase (GST) pulldown assays (250). Interaction was further demonstrated with coexpression of IGFBP-3 and GalNAc-T14 in human embryonic kidney cells and co-immunoprecipitation. GalNAc-T14 is probably involved in *O*-glycosylation of IGFBP-3; however, there are no predicted *O*-glycosylation sites on IGFBP-3. Therefore, the function of GalNAc-T14 is unknown and whether the interaction has biological significance remains to be determined.

Of clinical significance, study of children with congenital disorders of glycosylation (CGD) shows incomplete glycosylation of ALS and IGFBP-3 is associated with lower levels of IGF-1, IGF-2, IGFBP-3, and ALS compared with controls. Ternary complex formation of IGF, IGFBP-3, and ALS was impaired in CDG. Linear growth was improved with partial normalization of ALS and IGFBP-3 glycosylation, demonstrating probable functional significance of IGFBP-3 glycosylation (175).

Ubiquitination. The ubiquitin-protease system serves as a proteolytic degradation pathway for IGFBP-3. Ubiquitination is achieved through successive actions of three enzymes: E1, E2, and E3. E1 forms an ubiquitin-adenylate intermediate, acting as a bond between the COOH terminal carboxyl group of ubiquitin and the E1 active site; E2 is an ubiquitin-conjugating enzyme that attaches ubiquitin moieties to the target protein; E3 functions as the substrate recognition module that recruits the target, guides the transfer of the ubiquitin from E2 to the targeted protein's lysine residues allowing for elongation of the polyubiquitin chain (189). During ubiquitination, E3 recruits targeted proteins for degradation, E2 attaches the ubiquitin marker to the target protein, and E1 forms the bond between the ubiquitin and the E1 active site. Once the substrate is ubiquitin-tagged for proteolysis, the substrate is fed into the catalytic core for proteolysis and recycling (110).

IGFBP-3 is targeted to the ubiquitin-protease pathway, where degradation of IGFBP-3 is dependent on E1 as modeled in a cell line that expresses a temperature-sensitive mutant of ubiquitin-activating enzyme (ts20b) (207). Polyubiquitination of IGFBP-3 requires nuclear localization because cytosolic IGFBP-3 has a longer half-life compared with nuclear IGFBP-3, suggesting greater proteolytic degradation of nuclear IGFBP-3 (207). Results of this study demonstrated that nuclear IGFBP-3 is highly polyubiquitinated at multiple lysine residues and ubiquitination can

be inhibited by specific 26S protease inhibitors that stabilize nuclear IGFBP-3. Importantly, the molecular mechanism of IGFBP-3 inactivation by human papilloma virus E7 is inactivation of growth-inhibitory IGFBP-3 (167) is via interaction with the NLS sequence (208). Figure 5 summarizes factors that modulate IGFBP-3 transcription, availability and action.

IGFBP-3 ACTION IN CELLULAR AND WHOLE BODY MODELS

Cancer

The IGF-IGFBP system has been implicated in the pathogenesis and progression of cancer (154). Multiple epidemiological studies have examined the relationship between serum IGF, IGFBP-3, and cancer incidence; however, results are controversial and are attributed to non-standardized laboratory methods (6, 199, 204). The majority of empirical IGFBP-3 research has shown potentiation or inhibition of cell proliferation (61). IGFBP-3 controls IGF availability in which IGFBP-3 maintains IGF-IR sensitization or slowly releases IGF-1 to its receptor, resulting in a growth stimulatory effect. Or, IGFBP-3 may bind IGF to inhibit interaction with its receptor. Another explanation for this discrepant action involves disruption of cholesterol-stabilized membrane complexes or fibronectin receptor antibodies, which consistently reverses IGFBP-3 apoptosis on breast cancer cells in vitro (27). IGF-dependent models show that IGFBP-3 is capable of inhibiting growth through sequestration of IGF-1. Additionally, in vivo animal models suggest that IGFBP-3 suppresses tumor formation and growth inhibition independent of IGF. This section will focus on these pro- and anti-proliferative actions in cellular and animal models.

In vitro. IGF-DEPENDENT GROWTH STIMULATION. Although IGFBP-3 is most known for its growth inhibitory effects, IGFBP-3 has the capacity to maintain growth stimulatory effects. Preincubation of bovine fibroblasts with IGF-1 resulted in decreased IGF-R binding and subsequent decreased IGF-1- stimulated DNA synthesis (48). However, the addition of IGFBP-3 to this system during the preincubation blocked the decrement in receptor availability and maintained IGF-R sensitization. IGFBP-3 may enhance IGF-1 activity by slowly releasing IGF-1 to its receptor and inhibit IGF-R downregulation normally caused by high free IGF-1 exposure (94). Since IGF-1 has a lower affinity for proteolyzed IGFBP-3 than IGF-R, it may act to preserve IGF-IR sensitivity secondary to IGF-1 binding.

IGF-DEPENDENT GROWTH INHIBITION. IGFBP-3 inhibited cell proliferation in human promyelocytic leukemia cells, whereas an IGF-1 analog with reduced affinity for IGFBP-3, [des-(1-3)-IGF-1] failed to attenuate growth (155). Thus IGFBP-3 sequestration of IGF-1 prevented IGF-R binding while des-(1-3)-IGF-1 could bind to IGF-R to preserve growth. Similarly, IGFBP-3 inhibited estradiol production in human granulosa cells when stimulated by FSH and IGF-1, whereas des-(1-3)-IGF-1 failed to produce the same effect in vitro (16).

IGFBP-3 can also be used to inhibit cell growth in cells resistant to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (EGFR TKI). EGFR TKI blocked homo- and heterodimerization of phosphatidylinositol 3-kinase (PI3K) and Akt (63) to inhibit survival and cause apoptosis (231). However, some cancers acquire resistance to EGFR TKI (64). Thus IGFBP-3 can be used to inhibit activation of IGF-R and subsequent downstream activation of cell survival pathways. To illustrate this point, a cell line with wild-type EGFR gene amplification, A431, was used to induce acquired resistance to EGFR TKIs (A43-gefitinib resistant). IGFBPs are downregulated in A43-gefitinib resistant cells and immunoprecipitation of conditioned media, and subsequent incubation with ^{125}I-labeled IGF-1 demonstrated substantially more intense IGFBP-3 binding in parental (gefitinib-sensitive) versus gefitinib-resistant cells, indicating lack of IGFBP-3 may contribute to increased activation of IGF-R pathway in gefitinib-resistant cells (101). Reintroduction of IGFBP-3 reversed the resistant phenotype. Only combination treatment of recombinant IGFBP-3 and gefitinib resulted in marked inhibition on cell growth in A431-gefitinib-resistant cells, whereas either treatment alone had no effect on cell growth (101). Clinical trials conducted in patients with non-small cell lung cancer orally ingested EGFR TKI and this prolonged patient survival was prolonged with improvements in disease-related symptoms and tumor regression (82, 138, 216). Furthermore, combination treatment of gefitinib and IGF-R-specific monoclonal antibody treatment in

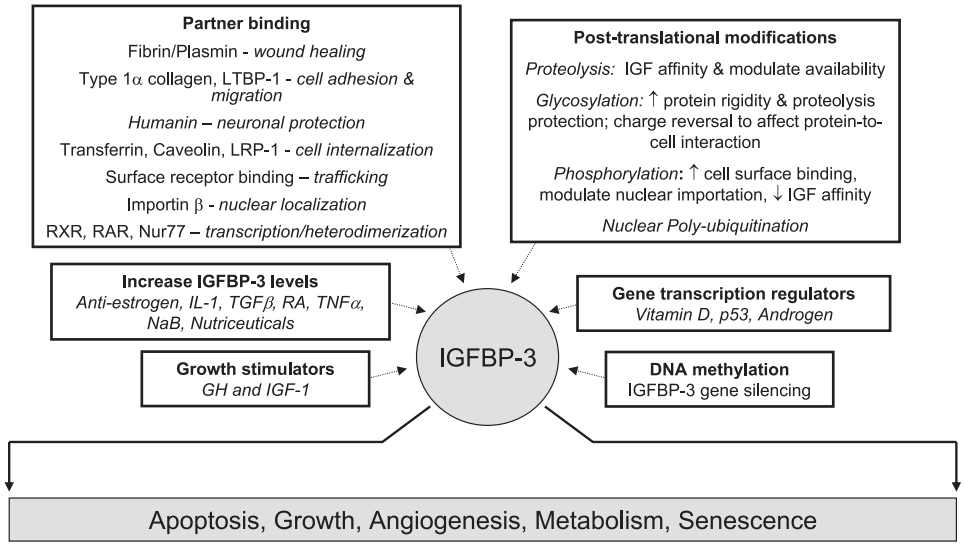

Fig. 5. An overview of the regulation of IGFBP-3 transcription, availability and action. ↑, increase; ↓, decrease; RA, retinoic acid; NaB, sodium butyrate.

mice with EGFR gene amplification prevented tumor recurrence, whereas either treatment alone failed to inhibit tumor growth. The role of IGFBP-3 has not been investigated in vivo and whether combination treatment of IGFBP-3 and gefitinib would produce results of similar amplitude remains unknown.

IGF-INDEPENDENT GROWTH INHIBITION. Evidence suggests that IGFBP-3 affects cell proliferation through IGF-independent mechanisms (183). These independent effects were first manifest by transfection of an IGFBP-3 overexpression vector into cells lacking IGF-R. Overexpression of IGFBP-3 in murine fibroblast cells with disrupted IGF-R gene resulted in growth inhibition, whereas no effect was observed in wild-type cells. These results indicate that IGFBP-3 acts independently from the IGF-R to inhibit cell proliferation (236). In a different study, a 16-kDa IGFBP-3 fragment with total loss of IGF affinity inhibited IGF-1-stimulated DNA synthesis in a chick embryo fibroblast assay to the same extent as intact IGFBP-3. In addition, a 22/25-kDa fragment with 50-fold decreased affinity for IGF-1 compared with intact rhIGFBP-3 also reduced IGF-1-stimulated DNA synthesis by 50%. The fragment with weak affinity for IGF-1 is therefore a weak antagonist of IGF action, whereas the fragment with no affinity for IGF was able to abrogate IGF-stimulated cell proliferation (143).

Recombinant IGFBP-3 treatment resulted in DNA fragmentation and a dose-dependent induction of apoptosis in a p53 negative prostate cancer cell line (PC-3) (197). Furthermore, IGF-R-negative fibroblast cells derived from an IGF-R knockout (KO) mouse were treated with exogenous IGFBP-3 and increased apoptosis. Transfection of IGFBP-3 into IGF-R-negative murine fibroblasts demonstrated that DNA fragmentation was more prevalent in cells transfected with IGFBP-3, and not surprising, a substantial increase in apoptosis occurred (197). Apoptosis was only partially suppressed with exogenous IGF-1, and apoptosis was unaffected by addition of long (L)R3-IGF-1, an IGF analog that does not bind IGFBP-3 (197). Taken together, these data suggest IGFBP-3 is capable of inducing apoptosis in an IGF-I-independent manner.

Similarly, IGFBP-3 treatment on undifferentiated chrondrocytes, a cell line that does not express IGF and IGFBP-3, resulted in a 40-fold increase in pro-apoptotic STAT-1 mRNA (232), whereas no increase in STAT-1 was observed with combination treatment of IGF-1 and IGFBP-3 or with IGF-1 alone. Upregulation of STAT-1 was also observed when chrondrocytes were treated with a mutant IGFBP-3 incapable of binding IGFs, showing IGF-independent regulation of STAT-1. An antisense STAT-1 ODN abolished IGFBP-3-induced apoptosis independent from IGF action. Moreover, demonstration of STAT-1 nuclear localization and phosphorylation in response to IGFBP-3 treatment provides additional evidence of IGF-independent action (232).

Another mechanism of presumed IGF independence involves IGFBP-3 activation of a phosphotyrosine phosphatase, indicating IGFBP-3 can inhibit IGF-R activation to attenuate mitogenic signaling (200). Normally, tyrosine kinase activity leads to tyrosine phosphorylation of IRS-1 and IRS-2 to initiate PI3K and MAPK. However, IGFBP-3 stimulated phosphotyrosine phosphatase, inhibiting IRS-1 and IRS-2 signaling, and thus suppresses cell proliferation (200). Additionally, GGG-IGFBP-3, a mutant lacking intrinsic IGF binding affinity, suppressed several key players in the apoptotic cascade, including the c-Raf-MEK-ERK pathway and p38 kinase in rat insulinoma cells in a dose- and time-dependent manner (40). These results suggest that IGFBP-3 inhibits cell growth through an IGF-independent pathway.

Data from a different study that employed a non-IGF binding IGFBP-3 mutant suggest that IGFBP-3 binding to a cell surface receptor or nuclear translocation into human breast cancer cells are not required for growth inhibition (28). Breast cancer cells (T47D) were infected with a mutant IGFBP-3-expressing adenovirus. These cells had reduced cell surface association and did not localize to the nucleus and still induced caspase-dependent apoptosis in T47D cells. Furthermore, incubation of mutant IGFBP-3 with vector-infected T47D cells with LR3-IGF-I had no significant effect on the ability of mutant IGFBP-3 to induce apoptosis. Thus this study showed that the IGFBP-3 mutant inhibited growth even with reduced cell surface binding and non-nuclear localization, suggesting IGFBP-3 may function in multiple pathways to inhibit growth in breast cancer cells (28) or that the mechanism of tumor suppression may be cell-type specific.

Apoptosis may also be mediated by IGFBP-3, RXRα, and Nur77. IGFBP-3 binds nuclear receptor RXRα, which is required for the apoptotic effects of IGFBP-3 (159, 161). RXRα heterodimerizes with orphan nuclear receptor Nur77 to assist translocation of Nur77 out of the nucleus. In the nucleus, Nur77 functions as a transcription factor to mediate cell proliferation, whereas in the mitochondria it mediates apoptosis. In fact, translocation of Nur77 into the mitochondria induced apoptosis in leukemia (260), lung (54), ovary (112), and colon cancer cells (246).

The relationship among IGFBP-3, RXRα, and Nur77 was confirmed utilizing $RXR\alpha^{+/+}$ and $RXR\alpha^{-/-}$ embryonic carcinoma cells. IGFBP-3 treatment reduced nuclear Nur77 in $RXR\alpha^{+/+}$ cells, consistent with Nur77 translocation from the nucleus to mitochondria, whereas nuclear Nur77 was increased in $RXR\alpha^{-/-}$ cells (150). Rapid mitochondrial translocation of Nur77 by IGFBP-3 was mediated in part by an IGF-R independent mechanism as IGFBP-3 still maintained its ability to induce apoptosis in IGF-R negative embryonic fibroblast cells.

A follow-up study confirmed that Nur77 and IGFBP-3 associate in the cytoplasmic compartment in prostate cancer cells (148). To illustrate that Nur77 translocation occurs in vivo, LAPC-4 xenografts were implanted in severe combined immunodeficiency (SCID) mice and after treatment with IGFBP-3 or saline, tumor sections were stained against Nur77. In tumors removed from mice treated with saline, Nur77 staining exhibited predominantly nuclear staining. However, tumors removed from mice treated with IGFBP-3 treatment had primarily cytoplasmic staining of Nur77. Collectively, these data present a role for IGFBP-3 as a multicompartmental signaling molecule.

IGF-INDEPENDENT GROWTH INHIBITION THROUGH OTHER EFFECTORS. IGF-independent mechanisms may also be regulated through other compounds. As reviewed earlier, RA (2, 97, 169, 215), TGF-β (168), TNF-α (196), and 1,25-OH_2D_3 (22, 170) can posttranscriptionally modify IGFBP-3 to potentiate its growth inhibitory effects. 1,25-OH_2D_3 can increase IGFBP-3 protein and mRNA levels through posttranslational gene regulation. Since addition of exogenous IGFBP-3 inhibited cell proliferation in LNCaP cells, it is interesting that antisense IGFBP-3 ODN or neutralizing IGFBP-3 antibodies abrogated the growth inhibitory effects of 1,25-OH_2D_3. IGFBP-3 upregu-

lated expression of p21/WAF1 protein to cause cell arrest, and IGFBP-3 neutralizing antibodies completely blocked 1,25-OH_2D_3 induced upregulation of the cyclin-dependent kinase inhibitory protein p21/WAF1. Thus these data suggest that IGFBP-3 is required for the anti-proliferative effects of 1,25-OH_2D_3 and p21/WAF1 may be responsible for the growth inhibitory effects (22).

IGFBP-3 is also able to modify the actions of these anti-proliferative agents. For example, treatment of ER-negative human breast cancer cells with RA or TGF-β_2 resulted in significant growth inhibition and a concomitant twofold increase in IGFBP-3 protein and mRNA versus untreated controls (97). Exogenous IGFBP-3 treatment alone inhibited cell growth by 40%. However, antisense IGFBP-3 ODN inhibited RA- and TGF-β_2-induced increase of IGFBP-3 protein and mRNA expression. As a result the anti-proliferative effects were attenuated with either treatment. Cell proliferation was not attenuated by sense IGFBP-3 ODN, suggesting that IGFBP-3 affects RA and TGF-β_2-associated growth inhibition (97).

Pro-inflammatory cytokines such as TNF-α and IL-1 stimulate apoptosis via induction of DNA fragmentation (58). Antisense IGFBP-3 ODN blocked TNF-α and IL-1-induced DNA fragmentation in rat insulinoma and hamster insulin-secreting tumor cells, respectively (217). In a different study, antisense IGFBP-3 ODN also inhibited TNF-α apoptosis and DNA fragmentation in mesangial cells (237). IGFBP-3 antisense enhanced Akt phosphorylation (Thr^{308}) while recombinant hIGFBP-3 inhibited Akt phosphorylation; this provides a mechanism by which IGFBP-3 inhibits cell survival.

TNF-α treatment increased IGFBP-3 expression in PC-3 cells in a dose- and time-dependent manner (196). The apoptotic response due to TNF-α was abolished with cotreatment of antisense IGFBP-3 ODN or IGFBP-3 neutralizing antibodies and inactivated survival protein Bcl-2. This indicated that IGFBP-3 and concomitant Bcl-2 inactivation is necessary for TNF-α-induced growth inhibition in PC-3 cells (196). These studies indicated that IGFBP-3 may regulate induction of pro-inflammatory cytokines and apoptosis in tumor cells.

Quercetin, a flavonoid found in grapes, green vegetables, and onions, induced apoptosis of PC-3 cells (240). This was accompanied with a decrease in IGF-1 and -2 and accumulation of IGFBP-3 in conditioned media. Also, anti-apoptotic Bcl-2 and Bcl-xL protein expression was significantly reduced, whereas pro-apoptotic Bax and caspase-3 expression were upregulated (240).

IGF-INDEPENDENT CELL SURVIVAL AND PROLIFERATION. The action of IGFBP-3 can be reversed with fibronectin and depletion of membrane cholesterol (27). IGFBP-3 normally induces apoptosis in breast cancer cells (Hs578T, MCF-7). However, IGFBP-3 action was reversed in non-IGF-responsive Hs578T cells when plated onto fibronectin such that IGFBP-3 enhanced cell survival and proliferation (171). Furthermore, when a specific antibody for the fibronectin receptor, α5β1, was applied used in C2-ceramide induced apoptosis, IGFBP-3 reversed apoptosis to promote cell survival (27). IGFBP-3 also reduced C2-induced apoptosis in the presence of filipin, an agent used to deplete membrane cholesterol. Similar results were observed in the presence of nystatin, an agent used to disrupt cholesterol-stabilized complexes. IGFBP-3 intracellular localization was increased in the presence of nystatin, indicating the reversal of the action of IGFBP-3 was not due to reduced IGFBP-3 entry into the cell.

In a different breast cancer cell line that has no detectable caveolin-1 (MCF-7), a SPD reversed C2-induced apoptosis in the presence of an agent that depletes membrane cholesterol MβCD (27). This finding was surprising because SPD (which lacks the region that interacts with transferrin) could affect MCF-7 cells even in the absence of caveolin-1. Caveolin-1 may facilitate actions of full-length IGFBP-3 but more interesting is the observation that fragments of IGFBP-3 can still exert similar actions. Furthermore, SPD action on cell proliferation is IGF independent because SPD is non-IGF binding. IGFBP-3 and its fragments such as SPD can either inhibit or promote cell survival; the action is dependent on the status of cholesterol-stabilized integrin receptor complexes (27).

In vivo. GROWTH INHIBITION. Mice bearing human prostate 22RV1 tumor xenografts were fed apigenin, a flavonoid present in parsley and celery (219). Daily oral intake of apigenin resulted in significant increases in tumor IGFBP-3 mRNA and apoptosis, concomitant with decreased tumor volume compared with control mice. An in vitro model was then employed to demonstrate that apigenin induces IGFBP-3. 22RV1 cells were treated with apigenin or an IGFBP-3 antisense ODN alone, or a combination of the two. Apigenin treatment resulted in a 41% decrease in the number of viable cells, increased apoptosis. Moreover, there was evidence of poly (ADP-ribose) polymerase cleavage, indicative of diminished DNA repair capacity. IGFBP-3 antisense ODN treatment resulted in a 9% increase in viable cells, and no evidence of poly (ADP-ribose) polymerase cleavage. The combination treatment reversed the apoptotic effects of apigenin by 31%, suggesting that growth inhibition caused by apigenin is related to IGFBP-3.

Continuous green tea polyphenol infusion for 24 wk substantially reduced IGF-1 and increased IGFBP-3 levels in the prostate of transgenic adenocarcinoma of the mouse prostate mice. Treatment was associated with attenuated protein expression of PI3K, phosphorylation of Akt, and significant inhibition of vascular endothelial growth factor (VEGF), uPA, MMP-2, and MMP-9, genes that promote angiogenesis (3). Similarly, dietary feeding of silibinin, the active component of flavonoid antioxidant silymarin (milk thistle extract) significantly inhibited tumor volume in DU145 tumor xenograft nude mice. Moreover, silibinin feeding before tumors were introduced also resulted in reduced tumor weight. These anticancer effects were attributed to increased plasma IGFBP-3 (224).

To examine effect of IGFBP-3 on angiogenesis, human CaP xenografts (22RV1) were established in SCID mice and mice were treated with either rhIGFBP-3 or saline over a 16-day period (160). There was a sevenfold increase in transferase-mediated nick end-labeling (TUNEL)-positive staining and caspase-3 staining in tumors of mice treated with IGFBP-3 versus mice treated with saline. CD31 staining in microvessels and endothelial cells was reduced by half in tumors treated with IGFPB-3 versus control, indicating IGFBP-3 suppresses intra-tumoral angiogenesis. Furthermore, cotreatment of IGFBP-3 and VEGF, a potent stimulator of vascular formation, inhibited VEGF-induced vessel formation in human endothelial cellular vessel formation in matrigel. Treatment with IGF-1 induced vascular formation, which is consistent with its angiogenic effects. Treatment with IGFBP-3 blocked the an-

giogenic effects of IGF-1 and treatment with LR3-IGF-1 partially blocked vascular formation, suggesting that a fraction of anti-angiogenic effects of IGFBP-3 were independent from IGF-1. In a different experiment, IGFBP-3 inhibited growth of new vascular vessels in chicken embryo chick allantoic membrane, illustrating that IGFBP-3 inhibits angiogenesis in vivo. The results of this study indicate that IGFBP-3 increases apoptosis and attenuates tumor growth and angiogenesis (160). These in vivo results are consistent with earlier in vitro work that showed IGFBP-3 induced rapid apoptosis in endothelial cells in an IGF-independent manner (79).

In contrast to the findings discussed above (160), Chang et al. (36) showed that IGFBP-3 exerts pro-angiogenic effects and promotes endothelial precursor cell migration in $CD34^+$ (but not $CD14^+$ monocytes), tube formation, and differentiation into endothelial cells. Injection of hematopoietic stem cells transfected with the plasmid-expressing IGFBP-3 into the vitreous humor of mouse pups attenuated vascular regression, increased vessel stabilization, and lead to quicker blood vessel development during hyperoxic insult. These angiogenic changes accounted for the reduced retinal ischemia in pups; these observations were absent in pups injected with an empty control vector. Pups treated with the IGFBP-3 plasmid showed early enhanced pro-angiogenesis and reduced anti-angiogenesis as measured with vascular blood vessel formation and preretinal neovascularization, respectively. Furthermore, pups treated with IGFBP-3-expressing plasmids had increased intraretinal vascular density and reduced preretinal vascular density in the same midperiphery region, perhaps indicating IGFBP-3 does not provide complete protection from hyperoxia-induced vessel regression or specificity of IGFBP-3 action.

Likewise, IGFBP-3 was also shown to stimulate neovessel formation in human endothelial cells (93). In this study, IGFBP-3 upregulated VEGF and matrix MMP-2 and- 9. The discrepancy in IGFBP-3 action on cell growth may be model and local environment specific. For example, IGFBP-3 exerts pro-apoptotic effects in carcinoma models, whereas "healthy" cells exposed to hyperoxic conditions and IGFBP-3 have enhanced angiogenesis, neovascularization, and cell survival. Indeed, utilizing IGFBP-3 KO mice, IGFBP-3 suppressed retinopathy through suppression of oxygen-induced vessel loss and promotion of vascular regrowth in a noncancer model (163). In addition, IGFBP-3 may have a role in hematopoietic stem cell and endothelial precursor cell function during vascular development (36).

IGF-INDEPENDENT GROWTH INHIBITION. To examine the effect of IGFBP-3 on tumor growth in vivo, SCID mice with LAPC4 xenografts were treated with either IGFBP-3, an RXR ligand (VTP 194204), a combination of the two, or saline for 3 wk (161). Although differences in gross tumor growth and apoptosis were absent or modest with either IGFBP-3 or the RXR ligand treatment, respectively, TUNEL assays indicated massive cell death with combination treatment. Combination treatment resulted in a 50% reduction in tumor growth and a 40% reduction in serum PSA levels. IGFBP-3 and RXR ligand in combination was essential to prevent tumor growth through induction of apoptosis (161). Interaction between RXR and IGFBP-3 presumably provides evidence for IGF-independent effects on tumor growth.

In another study, investigators aimed to differentiate whether the pro-apoptotic effects of IGFBP-3 are IGF dependent or independent. They bred the LPB-Tag mice (that have increased susceptibility to prostate cancer) with CMVBP-3 and PGKBP-3 mice (that have overexpression of IGFBP-3) and PGKmBP-3 (which express nonbinding IGF-1 mutant, I56G/L80G/L81G). The LPB-Tag mouse model represents the deletion of the simian virus 40 large T-antigen (Tag) under long probasin promoter (LPB), which is a murine cancer model that is androgen-related, prostate-specific, and of epithelial origin (131). The PGKBP-3 and CMVBP-3 model were developed by cloning human IGFBP-3 cNDA downstream of either the mouse phosphoglycerate (PGK) promoter or the cytomegalovirus (CMV) promoter, respectively, to result in IGFBP-3 overexpression (177). Whereas the I56G/L80G/L81G-mutant IGFBP-3 does not bind IGF-1, it still retains its IGF-independent apoptotic effects in vitro (220).

Overexpression of IGFBP-3 attenuated prostate tumor development in both LPB-Tag/PGKBP-3 and LPB-Tag/CMVBP-3 mice, with the latter having a greater effect (220). The rate of tumor growth was significantly lower in the LPB-Tag/CMVBP-3 mice versus LPB-Tag/PGKBP-3 mice. LBP-Tag/CMVBP-3 mice had greater transgene expression in prostate tissue compared with LBP-Tag/PGKBP-3 mice, but circulating IGFBP-3 levels were similar. Therefore, tumor growth retardation was mainly due to paracrine/autocrine effects of IGFBP-3 because LPB-Tag/CMVBP-3 mice had significantly slower tumor growth, higher transgene expression in prostate tissue, and similar circulating IGFBP-3 levels compared with LPB-Tag/PGKBP-3 mice (222). The LBP-Tag/PGKmBP-3 (non-IGF-binding transgenic) and LPB-Tag/WT had similar tumor growth. However, from 15 to 21 wk of age the PPB-Tag/PGKmBP-3 mice had a significant reduction in tumor growth. This suggests that early prostate tumor development in these mice is IGF-1 dependent and later development may be independent of IGF-1 (222).

Metabolism

In vitro. Chan et al. (35) showed that IGFBP-3 inhibited insulin-stimulated glucose uptake in 3T3-L1 adipocytes. The investigators believed it was most likely dependent on GLUT-4 translocation but independent of IGF-1 and IGF-R action. Exposure to insulin alone resulted in a threefold increase of GLUT-4 translocation in adipocytes. However, exposure to insulin in conjunction with IGFBP-3 or IGFBP-3 NLS mutant caused significant reductions in GLUT-4 translocation to the plasma membrane, 39% and 35%, respectively. Whereas IGFBP-3 did not affect tyrosine phosphorylation of the insulin receptor, IGFBP-3 reduced insulin-stimulated Thr^{308} phosphorylation of Akt (but not Ser^{473} phosphorylation). These observations suggest Akt may be involved in IGFBP-3 inhibition of GLUT-4 translocation and insulin-stimulated glucose uptake (35).

In a different study, IGFBP-3 was also shown to inhibit insulin-stimulated glucose uptake in 3T3-L1 adipocytes (133). However, in contrast to the previously mentioned study, IGFBP-3 inhibited insulin-receptor phosphorylation. In addition, TNF-α treatment induced endogenous production of IGFBP-3 in 3T3-L1 adipocytes, and IGFBP-3 inhibited adiponectin expression in mature adipocytes. This suggests that

during times of chronic inflammation and increased circulating TNF-α, endogenous production of IGFBP-3 would increase in adipocytes, resulting in a subsequent reduction in adiponectin production.

Treatment and cellular expression of IGFBP-3 inhibited adipocyte differentiation of 3T3-L1 fibroblasts, whereas an IGFBP-3 mutant unable to bind to nuclear receptors failed to elicit the same response (34). IGFBP-3 interfered with PPARγ-dependent adipocyte differentiation while the mutant that does not bind RXRα or PPARγ (a nuclear receptor known to dimerize with RXRα) did not block the ligand-induced transactivation of the PPAR response element. Using a His6 pull-down assay, IGFBP-3 bound strongly to His6-PPARγ, whereas the mutant failed to bind PPARγ. IGFBP-3 interaction with PPARγ was further demonstrated with coimmunoprecipitation using 3T3-L1 lysates, demonstrating that PPARγ is an in vitro binding partner of IGFBP-3 in adipocytes. IGFBP-3 was also shown to inhibit PPARγ heterodimerization with RXRα in vitro (34).

Whereas TNF-α increased IGFBP-3 production in 3T3-L1 adipocytes (133), TNF-α blocked IGFBP-3 secretion from primary human muscle myoblasts to reduce myoblast differentiation (76). In addition, IGFBP-3 secretion into conditioned media increased during differentiation of myoblasts to myotubes. IGFBP-3 antisense treatment reduced differentiation and IGFBP-3 secretion into conditioned media, suggesting IGFBP-3 is necessary for differentiation of myoblasts.

In vivo. Increased IGFBP-3 proteolysis has been implicated in noninsulin-dependent diabetes mellitus patients, indicating that IGFBP-3 may be a key modulator in metabolic disease (12). Although initial reports suggested that IGFBP-3 overexpression in transgenic mice resulted in selective organomegaly and had little effect on metabolism (178, 179), use of a different promoter to overexpress IGFBP-3 resulted in dramatic metabolic effects in mice (177). Human IGFBP-3 cDNA was subcloned downstream of the mouse phosphoglycerate (PGK) promoter or the cytomegalovirus (CMV) promoter and transgenic (Tg) mice overexpressing IGFBP-3 were generated by injecting transgenes into pronuclei of fertilized CD-1 zygotes (177). Tg mice exhibited ubiquitous hIGFBP-3 mRNA expression, whereas wild-type (WT) controls had no hIGFBP-3 mRNA expression (221).

Silha et al. (221) reported that both transgenic mouse strains were lighter at birth and at 12 wk when compared WT, which corresponded to decreased body length. Liver and epididymal fat weights were heavier in CMVBP-3 Tg mice (but not PGKBP-3) mice while brain and kidney weights were reduced. Although the opposite findings would be expected in IGFBP-3 knockout (KO) mice, heavier liver (and quadriceps) weights were reported in 8-wk-old IGFBP-3 KO mice while body weights and body lengths were greater in KO mice (253). In a follow-up study, Silha et al. (221) reported that fasting blood glucose levels were significantly elevated in CMBVP-3 and PGKBP-3 Tg versus control mice, with CMVBP-3 Tg mice having the highest fasting glucose levels. CMVBP-3 mice (but not PGKBP-3) also had higher basal insulin concentrations when compared with WT. Similarly, in response to intraperitoneal glucose stimulation, glucose tolerance tests demonstrated that Tg mice had greater glucose concentrations over 180 min when comparing the areas under the glucose concentration-time curves. Tritiated 2-deoxyglucose uptake was measured under basal conditions, and CMVBP-3 Tg mice had reduced clearance from the circulation versus WT mice, whereas uptake into skeletal muscle and adipose tissue were also reduced (221). This indicates that overexpression of IGFBP-3 decreases glucose/insulin sensitivity at the periphery; however whether central (hepatic) insulin resistance existed in this model is unknown. In a recent report, IGFBP-3 KO mice had no observed differences in glucose concentrations during a glucose tolerance test compared with WT mice (253). This may indicate that IGFBP-3 contribution to whole body metabolism is complex and may reflect a scenario where genetic deletion of IGFBP-3 along with overexpression of IGFBP-3 can contribute to a similar metabolic phenotype.

To show that IGFBP-3 affects peripheral insulin action through central mechanisms, IGFBP-3 was infused into the third ventricle of the hypothalamus in male rats (180). Intracerebroventricular (ICV) infusion of IGFBP-3 significantly blunted insulin action at the liver during a hyperinsulinemic clamp. Under hyperinsulinemic conditions, IGF-1 infusion decreased the contribution of glycogenolysis to hepatic glucose production, whereas IGFBP-3 infusion resulted in a significant increase in glycogenolysis. In a healthy liver, insulin stimulation should reduce hepatic glucose production and glycogenolysis. Since IGFBP-3 infusion caused an increase in glycogenolysis, this indicates IGFBP-3 is associated with hepatic insulin resistance. Furthermore, IGFBP-3 infusion significantly attenuated peripheral glucose uptake while IGF-1 infusion marginally increased peripheral glucose uptake under hyperinsulinemic clamp conditions in vivo. This is in accordance with Silha et al. (221) who showed that IGFBP-3 overexpression reduced glucose uptake in skeletal muscle and adipose tissue. Collectively, these data indicate IGFBP-3 is associated with hepatic insulin resistance and decreased peripheral glucose sensitivity. Balancing IGF-1 and IGFBP-3 actions are paramount in maintaining glucose homeostasis (180). Importantly, this publication demonstrated the presence of IGF-1, IGF-R, and IGFBP-3 transcripts in the rat hypothalamus utilizing RT-PCR. A more recent publication also localized IGFBP-3 in murine and human neurons that secrete hypocretin, a neuropeptide associated with narcolepsy (113).

Ning et al. (182) reported that the generation of an IGFBP-3 null mouse had no obvious metabolic phenotype. Therefore, they generated a triple KO mouse (IGFBP-3, -4, -5) to study the role of IGFBPs in metabolism. The triple KO mouse had reduced body weights at birth and at 9 wk of age and reduced gonadal fat pad and quadriceps muscle masses when expressed relative to their body weight compared with their WT counterparts. Since MAPK stimulates cell growth, differentiation, and survival (80), decreased muscle weight may be attributed to decreased MAPK activation. Basal glucose levels were reduced by about ~30% in triple KO mice; blood IGF-1 bioactivity and levels were reduced in triple KO mice.

The triple KO mice also had higher insulin levels after challenged with glucose, and the increased insulin levels persisted for 30 min postglucose injection. The mice had increased beta cell area, accounting for increased insulin secretion postglucose challenge, which the authors believed enhanced glucose clearance postglucose challenge. The triple KO mice had reduced IGF-1 levels and since IGF-1 can inhibit insulin secretion in vivo (145), this may explain the increased insulin secretion in the KO. The KO mice most likely had increased

insulin activation of its receptor, which results in increased PI3K activity and formation of phosphatidylinositol-3 phosphate, leading to increased glucose uptake (83). In addition, the triple KO mice tended to have higher levels of phosphorylated AKT levels compared with WT, which may have resulted from increased insulin binding activity to its receptor. Although insulin kinetics in the triple KO mice were different (greater insulin secretion) from WT, interestingly the insulin tolerance test revealed that insulin sensitivity was not different between the triple KO and WT mice. The authors concluded that the triple KO mouse had postnatal growth retardation and increased insulin levels secondary to increased beta cell area.

Other Aspects of IGFBP-3 Action

Neuronal protection. Humanin was identified as a binding partner of IGFBP-3, with binding involving the COOH-terminal domain of IGFBP-3 in a region distinct from IGF binding (125). The 24-amino acid peptide is involved in protecting neurons from death associated with Alzheimer's disease and amyloid-β cytotoxicity (108, 233). IGFBP-3 is associated with humanin and antagonized its prosurvival effects in A172 glioblastoma cells. Interestingly, addition of IGFBP-3 potentiated humanin's rescue ability in primary murine cortical neurons from amyloid beta cytotoxicity and cell death.

Erectile dysfunction. Systemic infusion of IGF-1 or insulin results in vasodilation (15) and since IGF-1 is produced locally (57), it may be more functionally important in regulating local blood flow. Streptozotocin-induced diabetes in rats resulted in increased IGFBP-3 gene expression in major pelvic ganglia (MPG) by twofold; this response was absent in controls (1). Insulin alone or insulin plus free oxygen scavenger treatments were used to inhibit gene expression in diabetic MPG. Furthermore, a 10-fold increase in IGFBP-3 mRNA and IGFBP-3 transcripts of ~3, 2.6, and 1.5 kb were detected in penile tissue of diabetic rats but were absent in controls. This increase in IGFBP-3 could be corrected by insulin alone or insulin plus free oxygen scavenger treatments. Also, intracavernous pressure and IGFBP-3 mRNA levels in MPG and penile tissues were inversely correlated, indicating IGFBP-3 may be reducing local IGF bioavailability and inhibiting vasodilation. Similar findings were reproduced by a different group who also used streptozotocin-induced diabetes to increase IGFBP-3 expression in rats (228). Moreover, increased IGFBP-3 protein was reported to be localized to the epithelium of the urethra, penile endothelium, and smooth muscle in the corpus cavernosum. Thus hyperglycemia in combination with increased IGFBP-3 expression may have a role in the development of erectile dysfunction.

CONCLUSIONS

It seems that the context in which IGFBP-3 was first described to inhibit IGF-1 action (132) has focused its function to a carrier protein. Since this initial observation, IGFBP physiological actions of IGFBP-3 have been extensively studied, including *1*) its endocrine and local regulation of IGF-1 availability and action on cell growth and metabolism, and *2*) promotion of apoptosis in cellular and animal models through both IGF-1-independent and -dependent pathways. The latter has led to the development of anti-cancer therapies that promote cellular apoptosis through regulation of gene transcriptional activity or co-administration of IGFBP-3 with preexisting therapies. IGFBP-3 binds to nuclear proteins including transcriptional factors. Because transcription factors have been localized to other intracellular organelles, this suggests that IGFBP-3 may also localize to other intracellular compartments and organelles. Studies showing cytosolic and nuclear localization of IGFBP-3 suggest that it has IGF-independent functions. Current research demonstrating IGFBP-3's IGF-independent roles in suppressing tumor formation and carcinoma cell growth have used autocrine/paracrine models. Although this in itself is not a novel concept, where local IGFBP-3 is able to modulate cell growth, the interplay between IGFBP-3 systemic and local regulation is a concept that may bear physiological significance. As IGFBP-3 has been shown to have a combination of IGF-dependent and -independent roles, this could perhaps reflect the interaction between the endocrine and autocrine/paracrine systems. To understand the role of IGFBP-3 in whole body physiology, the juxtaposition of systemic and local regulation of IGFBP-3 should be further investigated in both healthy and diseased models. Further exploration of IGFBP-3 employing this perspective may provide insight into the pathophysiology and therapy across a wide continuum of disease.

REFERENCES

1. **Abdelbaky TM, Brock GB, Huynh H.** Improvement of erectile function in diabetic rats by insulin: possible role of the insulin-like growth factor system. *Endocrinology* 139: 3143–3147, 1998.
2. **Adamo ML, Shao ZM, Lanau F, Chen JC, Clemmons DR, Roberts CT Jr, LeRoith D, and Fontana JA.** Insulin-like growth factor-I (IGF-I) and retinoic acid modulation of IGF-binding proteins (IGFBPs): IGFBP-2, -3, and -4 gene expression and protein secretion in a breast cancer cell line. *Endocrinology* 131: 1858–1866, 1992.
3. **Adhami VM, Siddiqui IA, Ahmad N, Gupta S, Mukhtar H.** Oral consumption of green tea polyphenols inhibits insulin-like growth factor-I-induced signaling in an autochthonous mouse model of prostate cancer. *Cancer Res* 64: 8715–8722, 2004.
4. **Agarwal C, Lambert A, Chandraratna RA, Rorke EA, Eckert RL.** Vitamin D regulates human ectocervical epithelial cell proliferation and insulin-like growth factor-binding protein-3 level. *Biol Reprod* 60: 567–572, 1999.
5. **Ahlsen M, Carlsson-Skwirut C, Jonsson AP, Cederlund E, Bergman T, Bang P.** A 30-kDa fragment of insulin-like growth factor (IGF) binding protein-3 in human pregnancy serum with strongly reduced IGF-I binding. *Cell Mol Life Sci* 64: 1870–1880, 2007.
6. **Ali O, Cohen P, Lee KW.** Epidemiology and biology of insulin-like growth factor binding protein-3 (IGFBP-3) as an anti-cancer molecule. *Horm Metab Res* 35: 726–733, 2003.
7. **Andreatta-Van Leyen S, Hembree JR, Eckert RL.** Regulation of insulin-like growth factor 1 binding protein 3 levels by epidermal growth factor and retinoic acid in cervical epithelial cells. *J Cell Physiol* 160: 265–274, 1994.
8. **Anwar A, Zahid AA, Scheidegger KJ, Brink M, Delafontaine P.** Tumor necrosis factor-alpha regulates insulin-like growth factor-1 and insulin-like growth factor binding protein-3 expression in vascular smooth muscle. *Circulation* 105: 1220–1225, 2002.
9. **Arnold JT, Le H, McFann KK, Blackman MR.** Comparative effects of DHEA vs. testosterone, dihydrotestosterone, and estradiol on proliferation and gene expression in human LNCaP prostate cancer cells. *Am J Physiol Endocrinol Metab* 288: E573–E584, 2005.
10. **Atadja P, Wong H, Garkavtsev I, Veillette C, Riabowol K.** Increased activity of p53 in senescing fibroblasts. *Proc Natl Acad Sci USA* 92: 8348–8352, 1995.
11. **Bail HJ, Kolbeck S, Lindner T, Dahne M, Weiler A, Windhagen HJ, Raun K, Skjaerbaek C, Flyvbjerg A, Orskov H, Haas NP, Raschke MJ.** The effect of growth hormone on insulin-like growth factor I and bone metabolism in distraction osteogenesis. *Growth Horm IGF Res* 11: 314–323, 2001.
12. **Bang P, Brismar K, Rosenfeld RG.** Increased proteolysis of insulin-like growth factor-binding protein-3 (IGFBP-3) in noninsulin-dependent di-

abetes mellitus serum, with elevation of a 29-kilodalton (kDa) glycosylated IGFBP-3 fragment contained in the approximately 130- to 150-kDa ternary complex. *J Clin Endocrinol Metab* 78: 1119–1127, 1994.

13. **Bang P, Fielder PJ.** Human pregnancy serum contains at least two distinct proteolytic activities with the ability to degrade insulin-like growth factor binding protein-3. *Endocrinology* 138: 3912–3917, 1997.
14. **Bang P, Nygren J, Carlsson-Skwirut C, Thorell A, Ljungqvist O.** Postoperative induction of insulin-like growth factor binding protein-3 proteolytic activity: relation to insulin and insulin sensitivity. *J Clin Endocrinol Metab* 83: 2509–2515, 1998.
15. **Baron AD, Brechtel-Hook G, Johnson A, Hardin D.** Skeletal muscle blood flow. A possible link between insulin resistance and blood pressure. *Hypertension* 21: 129–135, 1993.
16. **Barreca A, Artini PG, Cesarone A, Arvigo M, D'Ambrogio G, Genazzani AR, Giordano G, Minuto F.** Interrelationships between follicle stimulating hormone and the growth hormone–insulin-like growth factor–IGF-binding proteins axes in human granulosa cells in culture. *J Endocrinol Invest* 19: 35–42, 1996.
17. **Baxter RC, Martin JL, Beniac VA.** High molecular weight insulin-like growth factor binding protein complex. Purification and properties of the acid-labile subunit from human serum. *J Biol Chem* 264: 11843–11848, 1989.
18. **Blum WF, Ranke MB.** Insulin-like growth factor binding proteins (IGFBPs) with special reference to IGFBP-3. *Acta Paediatr Scand Suppl* 367: 55–62, 1990.
19. **Blum WF, Ranke MB, Kietzmann K, Gauggel E, Zeisel HJ, Bierich JR.** A specific radioimmunoassay for the growth hormone (GH)-dependent somatomedin-binding protein: its use for diagnosis of GH deficiency. *J Clin Endocrinol Metab* 70: 1292–1298, 1990.
20. **Boffa LC, Gruss RJ, Allfrey VG.** Manifold effects of sodium butyrate on nuclear function. Selective and reversible inhibition of phosphorylation of histones H1 and H2A and impaired methylation of lysine and arginine residues in nuclear protein fractions. *J Biol Chem* 256: 9612–9621, 1981.
21. **Booth BA, Boes M, Andress DL, Dake BL, Kiefer MC, Maack C, Linhardt RJ, Bar K, Caldwell EE, Weiler J, and et al.** IGFBP-3 and IGFBP-5 association with endothelial cells: role of C-terminal heparin binding domain. *Growth Regul* 5: 1–17, 1995.
22. **Boyle BJ, Zhao XY, Cohen P, Feldman D.** Insulin-like growth factor binding protein-3 mediates 1 alpha,25-dihydroxyvitamin d(3) growth inhibition in the LNCaP prostate cancer cell line through p21/WAF1. *J Urol* 165: 1319–1324, 2001.
23. **Bramani S, Song H, Beattie J, Tonner E, Flint DJ, Allan GJ.** Amino acids within the extracellular matrix (ECM) binding region (201–218) of rat insulin-like growth factor binding protein (IGFBP)-5 are important determinants in binding IGF-I. *J Mol Endocrinol* 23: 117–123, 1999.
24. **Buckbinder L, Talbott R, Seizinger BR, Kley N.** Gene regulation by temperature-sensitive p53 mutants: identification of p53 response genes. *Proc Natl Acad Sci USA* 91: 10640–10644, 1994.
25. **Buckbinder L, Talbott R, Velasco-Miguel S, Takenaka I, Faha B, Seizinger BR, Kley N.** Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature* 377: 646–649, 1995.
26. **Buckway CK, Wilson EM, Ahlsen M, Bang P, Oh Y, Rosenfeld RG.** Mutation of three critical amino acids of the N-terminal domain of IGF-binding protein-3 essential for high affinity IGF binding. *J Clin Endocrinol Metab* 86: 4943–4950, 2001.
27. **Burrows C, Holly JM, Laurence NJ, Vernon EG, Carter JV, Clark MA, McIntosh J, McCaig C, Winters ZE, Perks CM.** Insulin-like growth factor binding protein 3 has opposing actions on malignant and nonmalignant breast epithelial cells that are each reversible and dependent upon cholesterol-stabilized integrin receptor complexes. *Endocrinology* 147: 3484–3500, 2006.
28. **Butt AJ, Fraley KA, Firth SM, Baxter RC.** IGF-binding protein-3-induced growth inhibition and apoptosis do not require cell surface binding and nuclear translocation in human breast cancer cells. *Endocrinology* 143: 2693–2699, 2002.
29. **Campbell PG, Durham SK, Hayes JD, Suwanichkul A, Powell DR.** Insulin-like growth factor-binding protein-3 binds fibrinogen and fibrin. *J Biol Chem* 274: 30215–30221, 1999.
30. **Campbell PG, Durham SK, Suwanichkul A, Hayes JD, Powell DR.** Plasminogen binds the heparin-binding domain of insulin-like growth factor-binding protein-3. *Am J Physiol Endocrinol Metab* 275: E321–E331, 1998.
31. **Campbell PG, Novak JF, Yanosick TB, McMaster JH.** Involvement of the plasmin system in dissociation of the insulin-like growth factor-binding protein complex. *Endocrinology* 130: 1401–1412, 1992.
32. **Carducci MA, Nelson JB, Chan-Tack KM, Ayyagari SR, Sweatt WH, Campbell PA, Nelson WG, Simons JW.** Phenylbutyrate induces apoptosis in human prostate cancer and is more potent than phenylacetate. *Clin Cancer Res* 2: 379–387, 1996.
33. **Carr JM, Owens JA, Baudinette RV, Wallace JC.** Analysis of insulin-like growth factor binding proteins in the tammar wallaby, Macropus eugenii. *Gen Comp Endocrinol* 99: 50–59, 1995.
34. **Chan SS, Schedlich LJ, Twigg SM, Baxter RC.** Inhibition of adipocyte differentiation by insulin-like growth factor binding protein-3. *Am J Physiol Endocrinol Metab* 296: E654–E663, 2009.
35. **Chan SS, Twigg SM, Firth SM, Baxter RC.** Insulin-like growth factor binding protein-3 leads to insulin resistance in adipocytes. *J Clin Endocrinol Metab* 90: 6588–6595, 2005.
36. **Chang KH, Chan-Ling T, McFarland EL, Afzal A, Pan H, Baxter LC, Shaw LC, Caballero S, Sengupta N, Li Calzi S, Sullivan SM, Grant MB.** IGF binding protein-3 regulates hematopoietic stem cell and endothelial precursor cell function during vascular development. *Proc Natl Acad Sci USA* 104: 10595–10600, 2007.
37. **Chang YS, Kong G, Sun S, Liu D, El-Naggar AK, Khuri FR, Hong WK, Lee HY.** Clinical significance of insulin-like growth factor-binding protein-3 expression in stage I non-small cell lung cancer. *Clin Cancer Res* 8: 3796–3802, 2002.
38. **Chang YS, Wang L, Liu D, Mao L, Hong WK, Khuri FR, Lee HY.** Correlation between insulin-like growth factor-binding protein-3 promoter methylation and prognosis of patients with stage I non-small cell lung cancer. *Clin Cancer Res* 8: 3669–3675, 2002.
39. **Chen D, Li M, Luo J, Gu W.** Direct interactions between HIF-1 alpha and Mdm2 modulate p53 function. *J Biol Chem* 278: 13595–13598, 2003.
40. **Chen X, Ferry RJ Jr.** Novel actions of IGFBP-3 on intracellular signaling pathways of insulin-secreting cells. *Growth Horm IGF Res* 16: 41–48, 2006.
41. **Clemmons DR, Thissen JP, Maes M, Ketelslegers JM, Underwood LE.** Insulin-like growth factor-I (IGF-I) infusion into hypophysectomized or protein-deprived rats induces specific IGF-binding proteins in serum. *Endocrinology* 125: 2967–2972, 1989.
42. **Cobb LJ, Liu B, Lee KW, Cohen P.** Phosphorylation by DNA-dependent protein kinase is critical for apoptosis induction by insulin-like growth factor binding protein-3. *Cancer Res* 66: 10878–10884, 2006.
43. **Cohen P, Graves HC, Peehl DM, Kamarei M, Giudice LC, Rosenfeld RG.** Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. *J Clin Endocrinol Metab* 75: 1046–1053, 1992.
44. **Cohen P, Peehl DM, Graves HC, Rosenfeld RG.** Biological effects of prostate specific antigen as an insulin-like growth factor binding protein-3 protease. *J Endocrinol* 142: 407–415, 1994.
45. **Colon E, Svechnikov KV, Carlsson-Skwirut C, Bang P, Soder O.** Stimulation of steroidogenesis in immature rat Leydig cells evoked by interleukin-1alpha is potentiated by growth hormone and insulin-like growth factors. *Endocrinology* 146: 221–230, 2005.
46. **Colston KW, Perks CM, Xie SP, Holly JM.** Growth inhibition of both MCF-7 and Hs578T human breast cancer cell lines by vitamin D analogues is associated with increased expression of insulin-like growth factor binding protein-3. *J Mol Endocrinol* 20: 157–162, 1998.
47. **Conover CA, De Leon DD.** Acid-activated insulin-like growth factor-binding protein-3 proteolysis in normal and transformed cells. Role of cathepsin D. *J Biol Chem* 269: 7076–7080, 1994.
48. **Conover CA, Powell DR.** Insulin-like growth factor (IGF)-binding protein-3 blocks IGF-I-induced receptor down-regulation and cell desensitization in cultured bovine fibroblasts. *Endocrinology* 129: 710–716, 1991.
49. **Coverley JA, Martin JL, Baxter RC.** The effect of phosphorylation by casein kinase 2 on the activity of insulin-like growth factor-binding protein-3. *Endocrinology* 141: 564–570, 2000.
50. **Cwyfan Hughes S, Mason HD, Franks S, Holly JM.** Modulation of the insulin-like growth factor-binding proteins by follicle size in the human ovary. *J Endocrinol* 154: 35–43, 1997.
51. **Daughaday WH, Hall K, Raben MS, Salmon WD Jr, van den Brande JL, and van Wyk JJ.** Somatomedin: proposed designation for sulphation factor. *Nature* 235: 107, 1972.

52. **Davenport ML, Isley WL, Pucilowska JB, Pemberton LB, Lyman B, Underwood LE, Clemmons DR.** Insulin-like growth factor-binding protein-3 proteolysis is induced after elective surgery. *J Clin Endocrinol Metab* 75: 590–595, 1992.
53. **Davies SC, Wass JA, Ross RJ, Cotterill AM, Buchanan CR, Coulson VJ, Holly JM.** The induction of a specific protease for insulin-like growth factor binding protein-3 in the circulation during severe illness. *J Endocrinol* 130: 469–473, 1991.
54. **Dawson MI, Hobbs PD, Peterson VJ, Leid M, Lange CW, Feng KC, Chen G, Gu J, Li H, Kolluri SK, Zhang X, Zhang Y, Fontana JA.** Apoptosis induction in cancer cells by a novel analogue of 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid lacking retinoid receptor transcriptional activation activity. *Cancer Res* 61: 4723–4730, 2001.
55. **De Benedetti F, Alonzi T, Moretta A, Lazzaro D, Costa P, Poli V, Martini A, Ciliberto G, Fattori E.** Interleukin 6 causes growth impairment in transgenic mice through a decrease in insulin-like growth factor-I. A model for stunted growth in children with chronic inflammation. *J Clin Invest* 99: 643–650, 1997.
56. **De Benedetti F, Meazza C, Oliveri M, Pignatti P, Vivarelli M, Alonzi T, Fattori E, Garrone S, Barreca A, Martini A.** Effect of IL-6 on IGF binding protein-3: a study in IL-6 transgenic mice and in patients with systemic juvenile idiopathic arthritis. *Endocrinology* 142: 4818–4826, 2001.
57. **Delafontaine P, Lou H, Alexander RW.** Regulation of insulin-like growth factor I messenger RNA levels in vascular smooth muscle cells. *Hypertension* 18: 742–747, 1991.
58. **Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG, Eizirik DL.** Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* 138: 2610–2614, 1997.
59. **Dinarello CA, Savage N.** Interleukin-1 and its receptor. *Crit Rev Immunol* 9: 1–20, 1989.
60. **Dobson ME, Ingram VM.** In vitro transcription of chromatin containing histones hyperacetylated in vivo. *Nucleic Acids Res* 8: 4201–4219, 1980.
61. **Duan C, Xu Q.** Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. *Gen Comp Endocrinol* 142: 44–52, 2005.
62. **el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B.** Definition of a consensus binding site for p53. *Nat Genet* 1: 45–49, 1992.
63. **Engelman JA.** The role of phosphoinositide 3-kinase pathway inhibitors in the treatment of lung cancer. *Clin Cancer Res* 13: s4637–4640, 2007.
64. **Engelman JA, Janne PA.** Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. *Clin Cancer Res* 14: 2895–2899, 2008.
65. **Fanayan S, Firth SM, Baxter RC.** Signaling through the Smad pathway by insulin-like growth factor-binding protein-3 in breast cancer cells. Relationship to transforming growth factor-beta 1 signaling. *J Biol Chem* 277: 7255–7261, 2002.
66. **Fanayan S, Firth SM, Butt AJ, Baxter RC.** Growth inhibition by insulin-like growth factor-binding protein-3 in T47D breast cancer cells requires transforming growth factor-beta (TGF-beta) and the type II TGF-beta receptor. *J Biol Chem* 275: 39146–39151, 2000.
67. **Feldser D, Agani F, Iyer NV, Pak B, Ferreira G, Semenza GL.** Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. *Cancer Res* 59: 3915–3918, 1999.
68. **Fernihough JK, Billingham ME, Cwyfan-Hughes S, Holly JM.** Local disruption of the insulin-like growth factor system in the arthritic joint. *Arthritis Rheum* 39: 1556–1565, 1996.
69. **Fernihough JK, Innes JF, Billingham ME, Holly JM.** Changes in the local regulation of insulin-like growth factors I and II and insulin-like growth factor-binding proteins in osteoarthritis of the canine stifle joint secondary to cruciate ligament rupture. *Vet Surg* 32: 313–323, 2003.
70. **Firth SM, Baxter RC.** Cellular actions of the insulin-like growth factor binding proteins. *Endocrinol Rev* 23: 824–854, 2002.
71. **Firth SM, Baxter RC.** Characterisation of recombinant glycosylation variants of insulin-like growth factor binding protein-3. *J Endocrinol* 160: 379–387, 1999.
72. **Firth SM, Baxter RC.** The role of glycosylation in the action of IGFBP-3. *Prog Growth Factor Res* 6: 223–229, 1995.
73. **Firth SM, Ganeshprasad U, Baxter RC.** Structural determinants of ligand and cell surface binding of insulin-like growth factor-binding protein-3. *J Biol Chem* 273: 2631–2638, 1998.
74. **Fontana JA, Burrows-Mezu A, Clemmons DR, LeRoith D.** Retinoid modulation of insulin-like growth factor-binding proteins and inhibition of breast carcinoma proliferation. *Endocrinology* 128: 1115–1122, 1991.
75. **Fortier LA, Kornatowski MA, Mohammed HO, Jordan MT, O'Cain LC, Stevens WB.** Age-related changes in serum insulin-like growth factor-I, insulin-like growth factor-I binding protein-3 and articular cartilage structure in Thoroughbred horses. *Equine Vet J* 37: 37–42, 2005.
76. **Foulstone EJ, Savage PB, Crown AL, Holly JM, Stewart CE.** Role of insulin-like growth factor binding protein-3 (IGFBP-3) in the differentiation of primary human adult skeletal myoblasts. *J Cell Physiol* 195: 70–79, 2003.
77. **Fowlkes JL, Serra DM.** Characterization of glycosaminoglycan-binding domains present in insulin-like growth factor-binding protein-3. *J Biol Chem* 271: 14676–14679, 1996.
78. **Fowlkes JL, Suzuki K, Nagase H, Thrailkill KM.** Proteolysis of insulin-like growth factor binding protein-3 during rat pregnancy: a role for matrix metalloproteinases. *Endocrinology* 135: 2810–2813, 1994.
79. **Franklin SL, Ferry RJ Jr, Cohen P.** Rapid insulin-like growth factor (IGF)-independent effects of IGF binding protein-3 on endothelial cell survival. *J Clin Endocrinol Metab* 88: 900–907, 2003.
80. **Freeman SM, Whartenby KA.** The role of the mitogen-activated protein kinase cellular signaling pathway in tumor cell survival and apoptosis. *Drug News Perspect* 17: 237–242, 2004.
81. **Froesch ER, Zapf J, Audhya TK, Ben-Porath E, Segen BJ, Gibson KD.** Nonsuppressible insulin-like activity and thyroid hormones: major pituitary-dependent sulfation factors for chick embryo cartilage. *Proc Natl Acad Sci USA* 73: 2904–2908, 1976.
82. **Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, Nishiwaki Y, Vansteenkiste J, Kudoh S, Rischin D, Eek R, Horai T, Noda K, Takata I, Smit E, Averbuch S, Macleod A, Feyereislova A, Dong RP, Baselga J.** Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. *J Clin Oncol* 21: 2237–2246, 2003.
83. **Funaki M, Randhawa P, Janmey PA.** Separation of insulin signaling into distinct GLUT4 translocation and activation steps. *Mol Cell Biol* 24: 7567–7577, 2004.
84. **Galanis M, Firth SM, Bond J, Nathanielsz A, Kortt AA, Hudson PJ, Baxter RC.** Ligand-binding characteristics of recombinant amino- and carboxyl-terminal fragments of human insulin-like growth factor-binding protein-3. *J Endocrinol* 169: 123–133, 2001.
85. **Galbiati F, Razani B, Lisanti MP.** Emerging themes in lipid rafts and caveolae. *Cell* 106: 403–411, 2001.
86. **Gatford KL, Dalitz PA, Cock ML, Harding R, Owens JA.** Acute ethanol exposure in pregnancy alters the insulin-like growth factor axis of fetal and maternal sheep. *Am J Physiol Endocrinol Metab* 292: E494–E500, 2007.
87. **Gelmann EP.** Molecular biology of the androgen receptor. *J Clin Oncol* 20: 3001–3015, 2002.
88. **Gibson CA, Staley MD, Baumrucker CR.** Identification of IGF binding proteins in bovine milk and the demonstration of IGFBP-3 synthesis and release by bovine mammary epithelial cells. *J Anim Sci* 77: 1547–1557, 1999.
89. **Glasscock GF, Hein AN, Miller JA, Hintz RL, Rosenfeld RG.** Effects of continuous infusion of insulin-like growth factor I and II, alone and in combination with thyroxine or growth hormone, on the neonatal hypophysectomized rat. *Endocrinology* 130: 203–210, 1992.
90. **Goda N, Tenno T, Inomata K, Shirakawa M, Tanaka T, Hiroaki H.** Intracellular protein delivery activity of peptides derived from insulin-like growth factor binding proteins 3 and 5. *Exp Cell Res* 314: 2352–2361, 2008.
91. **Goldstein S, Moerman EJ, Jones RA, Baxter RC.** Insulin-like growth factor binding protein 3 accumulates to high levels in culture medium of senescent and quiescent human fibroblasts. *Proc Natl Acad Sci USA* 88: 9680–9684, 1991.
92. **Goossens K, Esquenet M, Swinnen JV, Manin M, Rombauts W, Verhoeven G.** Androgens decrease and retinoids increase the expression of insulin-like growth factor-binding protein-3 in LNcaP prostatic adenocarcinoma cells. *Mol Cell Endocrinol* 155: 9–18, 1999.
93. **Granata R, Trovato L, Lupia E, Sala G, Settanni F, Camussi G, Ghidoni R, Ghigo E.** Insulin-like growth factor binding protein-3 induces angiogenesis through IGF-I- and SphK1-dependent mechanisms. *J Thromb Haemost* 5: 835–845, 2007.

94. **Grimberg A, Cohen P.** Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. *J Cell Physiol* 183: 1–9, 2000.
95. **Grimberg A, Coleman CM, Burns TF, Himelstein BP, Koch CJ, Cohen P, and El-Deiry WS.** p53-Dependent and p53-independent induction of insulin-like growth factor binding protein-3 by deoxyribonucleic acid damage and hypoxia. *J Clin Endocrinol Metab* 90: 3568–3574, 2005.
96. **Gucev ZS, Oh Y, Kelley KM, Labarta JI, Vorwerk P, Rosenfeld RG.** Evidence for insulin-like growth factor (IGF)-independent transcriptional regulation of IGF binding protein-3 by growth hormone in SKHEP-1 human hepatocarcinoma cells. *Endocrinology* 138: 1464–1470, 1997.
97. **Gucev ZS, Oh Y, Kelley KM, Rosenfeld RG.** Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor beta2-induced growth inhibition in human breast cancer cells. *Cancer Res* 56: 1545–1550, 1996.
98. **Guevara-Aguirre J, Vasconez O, Martinez V, Martinez AL, Rosenbloom AL, Diamond FB Jr, Gargosky SE, Nonoshita L, and Rosenfeld RG.** A randomized, double blind, placebo-controlled trial on safety and efficacy of recombinant human insulin-like growth factor-I in children with growth hormone receptor deficiency. *J Clin Endocrinol Metab* 80: 1393–1398, 1995.
99. **Gui Y, Murphy LJ.** Insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3) binds to fibronectin (FN): demonstration of IGF-I/IGFBP-3/fn ternary complexes in human plasma. *J Clin Endocrinol Metab* 86: 2104–2110, 2001.
100. **Gui Y, Murphy LJ.** Interaction of insulin-like growth factor binding protein-3 with latent transforming growth factor-beta binding protein-1. *Mol Cell Biochem* 250: 189–195, 2003.
101. **Guix M, Faber AC, Wang SE, Olivares MG, Song Y, Qu S, Rinehart C, Seidel B, Yee D, Arteaga CL, Engelman JA.** Acquired resistance to EGFR tyrosine kinase inhibitors in cancer cells is mediated by loss of IGF-binding proteins. *J Clin Invest* 118: 2609–2619, 2008.
102. **Guler HP, Zapf J, Schmid C, Froesch ER.** Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. *Acta Endocrinol* 121: 753–758, 1989.
103. **Han GR, Dohi DF, Lee HY, Rajah R, Walsh GL, Hong WK, Cohen P, Kurie JM.** All-trans-retinoic acid increases transforming growth factor-beta2 and insulin-like growth factor binding protein-3 expression through a retinoic acid receptor-alpha-dependent signaling pathway. *J Biol Chem* 272: 13711–13716, 1997.
104. **Hanafusa T, Yumoto Y, Nouso K, Nakatsukasa H, Onishi T, Fujikawa T, Taniyama M, Nakamura S, Uemura M, Takuma Y, Yumoto E, Higashi T, Tsuji T.** Reduced expression of insulin-like growth factor binding protein-3 and its promoter hypermethylation in human hepatocellular carcinoma. *Cancer Lett* 176: 149–158, 2002.
105. **Hardouin S, Gourmelen M, Noguiez P, Seurin D, Roghani M, Le Bouc Y, Povoa G, Merimee TJ, Hossenlopp P, Binoux M.** Molecular forms of serum insulin-like growth factor (IGF)-binding proteins in man: relationships with growth hormone and IGFs and physiological significance. *J Clin Endocrinol Metab* 69: 1291–1301, 1989.
106. **Harms KL, Chen X.** The C terminus of p53 family proteins is a cell fate determinant. *Mol Cell Biol* 25: 2014–2030, 2005.
107. **Hasegawa Y, Hasegawa T, Aso T, Kotoh S, Tsuchiya Y, Nose O, Ohyama Y, Araki K, Tanaka T, Saisyo S, and et al.** Comparison between insulin-like growth factor-I (IGF-I) and IGF binding protein-3 (IGFBP-3) measurement in the diagnosis of growth hormone deficiency. *Endocr J* 40: 185–190, 1993.
108. **Hashimoto Y, Tsuji O, Niikura T, Yamagishi Y, Ishizaka M, Kawasumi M, Chiba T, Kanekura K, Yamada M, Tsukamoto E, Kouyama K, Terashita K, Aiso S, Lin A, Nishimoto I.** Involvement of c-Jun N-terminal kinase in amyloid precursor protein-mediated neuronal cell death. *J Neurochem* 84: 864–877, 2003.
109. **Hembree JR, Agarwal C, Beard RL, Chandraratna RA, Eckert R.** Retinoid X receptor-specific retinoids inhibit the ability of retinoic acid receptor-specific retinoids to increase the level of insulin-like growth factor binding protein-3 in human ectocervical epithelial cells. *Cancer Res* 56: 1794–1799, 1996.
110. **Hershko A, Ciechanover A.** The ubiquitin system. *Annu Rev Biochem* 67: 425–479, 1998.
111. **Hoeck WG, Mukku VR.** Identification of the major sites of phosphorylation in IGF binding protein-3. *J Cell Biochem* 56: 262–273, 1994.
112. **Holmes WF, Soprano DR, Soprano KJ.** Comparison of the mechanism of induction of apoptosis in ovarian carcinoma cells by the conformationally restricted synthetic retinoids CD437 and 4-HPR. *J Cell Biochem* 89: 262–278, 2003.
113. **Honda M, Eriksson KS, Zhang S, Tanaka S, Lin L, Salehi A, Hesla PE, Maehlen J, Gaus SE, Yanagisawa M, Sakurai T, Taheri S, Tsuchiya K, Honda Y, Mignot E.** IGFBP3 colocalizes with and regulates hypocretin (orexin). *PLoS ONE* 4: e4254, 2009.
114. **Honegger B, Galic M, Kohler K, Wittwer F, Brogiolo W, Hafen E, Stocker H.** Imp-L2, a putative homolog of vertebrate IGF-binding protein 7, counteracts insulin signaling in Drosophila and is essential for starvation resistance. *J Biol* 7: 10, 2008.
115. **Hong J, Zhang G, Dong F, Rechler MM.** Insulin-like growth factor (IGF)-binding protein-3 mutants that do not bind IGF-I or IGF-II stimulate apoptosis in human prostate cancer cells. *J Biol Chem* 277: 10489–10497, 2002.
116. **Hossenlopp P, Segovia B, Lassarre C, Roghani M, Bredon M, Binoux M.** Evidence of enzymatic degradation of insulin-like growth factor-binding proteins in the 150K complex during pregnancy. *J Clin Endocrinol Metab* 71: 797–805, 1990.
117. **Huang SS, Leal SM, Chen CL, Liu IH, Huang JS.** Identification of insulin receptor substrate proteins as key molecules for the TbetaR-V/LRP-1-mediated growth inhibitory signaling cascade in epithelial and myeloid cells. *FASEB J* 18: 1719–1721, 2004.
118. **Huang SS, Ling TY, Tseng WF, Huang YH, Tang FM, Leal SM, Huang JS.** Cellular growth inhibition by IGFBP-3 and TGF-beta1 requires LRP-1. *FASEB J* 17: 2068–2081, 2003.
119. **Hughes SC, Xu S, Fernihough J, Hampton A, Mason HD, Franks S, van der Stappen J, Donnelly MJ, Holly JM.** Tissue IGFBP-3 proteolysis: contrasting pathophysiology to that in the circulation. *Prog Growth Factor Res* 6: 293–299, 1995.
120. **Hung H, Pollak M.** Regulation of IGFBP-3 expression in breast cancer cells and uterus by estradiol and antiestrogens: correlations with effects on proliferation: a review. *Prog Growth Factor Res* 6: 495–501, 1995.
121. **Huynh H, Pollak M, Zhang JC.** Regulation of insulin-like growth factor (IGF) II and IGF binding protein 3 autocrine loop in human PC-3 prostate cancer cells by vitamin D metabolite 1,25(OH)2D3 and its analog EB1089. *Int J Oncol* 13: 137–143, 1998.
122. **Huynh H, Yang X, Pollak M.** Estradiol and antiestrogens regulate a growth inhibitory insulin-like growth factor binding protein 3 autocrine loop in human breast cancer cells. *J Biol Chem* 271: 1016–1021, 1996.
123. **Hwa V, Oh Y, Rosenfeld RG.** Insulin-like growth factor binding protein-3 and -5 are regulated by transforming growth factor-beta and retinoic acid in the human prostate adenocarcinoma cell line PC-3. *Endocrine* 6: 235–242, 1997.
124. **Iannuzzi L, Di Meo GP, Perucatti A, Schibler L, Incarnato D, Gallagher D, Eggen A, Ferretti L, Cribiu EP, Womack J.** The river buffalo (Bubalus bubalis, 2*n* = 50) cytogenetic map: assignment of 64 loci by fluorescence in situ hybridization and R-banding. *Cytogenet Genome Res* 102: 65–75, 2003.
125. **Ikonen M, Liu B, Hashimoto Y, Ma L, Lee KW, Niikura T, Nishimoto I, Cohen P.** Interaction between the Alzheimer's survival peptide humanin and insulin-like growth factor-binding protein 3 regulates cell survival and apoptosis. *Proc Natl Acad Sci USA* 100: 13042–13047, 2003.
126. **Issa JP.** Methylation and prognosis: of molecular clocks and hypermethylator phenotypes. *Clin Cancer Res* 9: 2879–2881, 2003.
127. **Jaques G, Noll K, Wegmann B, Witten S, Kogan E, Radulescu RT, Havemann K.** Nuclear localization of insulin-like growth factor binding protein 3 in a lung cancer cell line. *Endocrinology* 138: 1767–1770, 1997.
128. **Jonsson CK, Setchell BP, Martinelle N, Svechnikov K, Soder O.** Endotoxin-induced interleukin 1 expression in testicular macrophages is accompanied by downregulation of the constitutive expression in Sertoli cells. *Cytokine* 14: 283–288, 2001.
129. **Kalus W, Zweckstetter M, Renner C, Sanchez Y, Georgescu J, Grol M, Demuth D, Schumacher R, Dony C, Lang K, Holak TA.** Structure of the IGF-binding domain of the insulin-like growth factor-binding protein-5 (IGFBP-5): implications for IGF and IGF-I receptor interactions. *EMBO J* 17: 6558–6572, 1998.
130. **Kanety H, Karasik A, Klinger B, Silbergeld A, Laron Z.** Long-term treatment of Laron type dwarfs with insulin-like growth factor-1 increases serum insulin-like growth factor-binding protein-3 in the absence of growth hormone activity. *Acta Endocrinol* 128: 144–149, 1993.
131. **Kasper S, Sheppard PC, Yan Y, Pettigrew N, Borowsky AD, Prins GS, Dodd JG, Duckworth ML, Matusik RJ.** Development, progres-

sion, and androgen-dependence of prostate tumors in probasin-large T antigen transgenic mice: a model for prostate cancer. *Lab Invest* 78: 319–333, 1998.

132. **Kaufmann U, Zapf J, Torretti B, Froesch ER.** Demonstration of a specific serum carrier protein of nonsuppressible insulin-like activity in vivo. *J Clin Endocrinol Metab* 44: 160–166, 1977.
133. **Kim HS, Ali O, Shim M, Lee KW, Vuguin P, Muzumdar R, Barzilai N, Cohen P.** Insulin-like growth factor binding protein-3 induces insulin resistance in adipocytes in vitro and in rats in vivo. *Pediatr Res* 61: 159–164, 2007.
134. **Kim ST, Lim DS, Canman CE, Kastan MB.** Substrate specificities and identification of putative substrates of ATM kinase family members. *J Biol Chem* 274: 37538–37543, 1999.
135. **Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R, Dickson RB.** Evidence that transforming growth factor-beta is a hormonally regulated negative growth factor in human breast cancer cells. *Cell* 48: 417–428, 1987.
136. **Kojic LD, Wiseman SM, Ghaidi F, Joshi B, Nedev H, Saragovi HU, Nabi IR.** Raft-dependent endocytosis of autocrine motility factor/phosphoglucose isomerase: a potential drug delivery route for tumor cells. *PLoS ONE* 3: e3597, 2008.
137. **Kojima S, Mulholland DJ, Ettinger S, Fazli L, Nelson CC, Gleave ME.** Differential regulation of IGFBP-3 by the androgen receptor in the lineage-related androgen-dependent LNCaP and androgen-independent C4-2 prostate cancer models. *Prostate* 66: 971–986, 2006.
138. **Kris MG, Natale RB, Herbst RS, Lynch TJ Jr, Prager D, Belani CP, Schiller JH, Kelly K, Spiridonidis H, Sandler A, Albain KS, Cella D, Wolf MK, Averbuch SD, Ochs JJ, and Kay AC.** Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA* 290: 2149–2158, 2003.
139. **Krishnan AV, Peehl DM, Feldman D.** Inhibition of prostate cancer growth by vitamin D: Regulation of target gene expression. *J Cell Biochem* 88: 363–371, 2003.
140. **Kruh J.** Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Mol Cell Biochem* 42: 65–82, 1982.
141. **Lalou C, Lassarre C, Binoux M.** Isolation and characterization of proteolytic fragments of insulin-like growth factor-binding protein-3. *Horm Res* 45: 156–159, 1996.
142. **Lalou C, Lassarre C, Binoux M.** A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails to bind IGF is a cell growth inhibitor. *Prog Growth Factor Res* 6: 311–316, 1995.
143. **Lalou C, Lassarre C, Binoux M.** A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails to bind IGFs inhibits the mitogenic effects of IGF-I and insulin. *Endocrinology* 137: 3206–3212, 1996.
144. **Laron Z, Klinger B, Blum WF, Silbergeld A, Ranke MB.** *IGF binding protein 3 in patients with Laron type dwarfism: effect of exogenous rIGF-I. Clin Endocrinol (Oxf)* 36: 301–304, 1992.
145. **Leahy JL, Vandekerkhove KM.** Insulin-like growth factor-I at physiological concentrations is a potent inhibitor of insulin secretion. *Endocrinology* 126: 1593–1598, 1990.
146. **Leal SM, Liu Q, Huang SS, Huang JS.** The type V transforming growth factor beta receptor is the putative insulin-like growth factor-binding protein 3 receptor. *J Biol Chem* 272: 20572–20576, 1997.
147. **Lee C, Sutkowski DM, Sensibar JA, Zelner D, Kim I, Amsel I, Shaw N, Prins GS, Kozlowski JM.** Regulation of proliferation and production of prostate-specific antigen in androgen-sensitive prostatic cancer cells, LNCaP, by dihydrotestosterone. *Endocrinology* 136: 796–803, 1995.
148. **Lee KW, Cobb LJ, Paharkova-Vatchkova V, Liu B, Milbrandt J, Cohen P.** Contribution of the orphan nuclear receptor Nur77 to the apoptotic action of IGFBP-3. *Carcinogenesis* 28: 1653–1658, 2007.
149. **Lee KW, Liu B, Ma L, Li H, Bang P, Koeffler HP, Cohen P.** Cellular internalization of insulin-like growth factor binding protein-3: distinct endocytic pathways facilitate re-uptake and nuclear localization. *J Biol Chem* 279: 469–476, 2004.
150. **Lee KW, Ma L, Yan X, Liu B, Zhang XK, Cohen P.** Rapid apoptosis induction by IGFBP-3 involves an insulin-like growth factor-independent nucleomitochondrial translocation of RXRalpha/Nur77. *J Biol Chem* 280: 16942–16948, 2005.
151. **Lee SH, Takahashi M, Honke K, Miyoshi E, Osumi D, Sakiyama H, Ekuni A, Wang X, Inoue S, Gu J, Kadomatsu K, Taniguchi N.** Loss of core fucosylation of low-density lipoprotein receptor-related protein-1 impairs its function, leading to the upregulation of serum levels of insulin-like growth factor-binding protein 3 in Fut8−/− mice. *J Biochem* 139: 391–398, 2006.
152. **Lelbach A, Scharf JG, Ramadori G.** Regulation of insulin-like growth factor-I and of insulin-like growth factor binding protein-1, -3 and -4 in cocultures of rat hepatocytes and Kupffer cells by interleukin-6. *J Hepatol* 35: 558–567, 2001.
153. **Lemmey AB, Glassford J, Flick-Smith HC, Holly JM, Pell JM.** Differential regulation of tissue insulin-like growth factor-binding protein (IGFBP)-3, IGF-I and IGF type 1 receptor mRNA levels, and serum IGF-I and IGFBP concentrations by growth hormone and IGF-I. *J Endocrinol* 154: 319–328, 1997.
154. **LeRoith D, Roberts CT Jr.** The insulin-like growth factor system and cancer. *Cancer Lett* 195: 127–137, 2003.
155. **Li Q, Li B, Wang X, Leri A, Jana KP, Liu Y, Kajstura J, Baserga R, Anversa P.** Overexpression of insulin-like growth factor-1 in mice protects from myocyte death after infarction, attenuating ventricular dilation, wall stress, and cardiac hypertrophy. *J Clin Invest* 100: 1991–1999, 1997.
156. **Li W, Fawcett J, Widmer HR, Fielder PJ, Rabkin R, Keller GA.** Nuclear transport of insulin-like growth factor-I and insulin-like growth factor binding protein-3 in opossum kidney cells. *Endocrinology* 138: 1763–1766, 1997.
157. **Li Y, Xiang J, Duan C.** Insulin-like growth factor-binding protein-3 plays an important role in regulating pharyngeal skeleton and inner ear formation and differentiation. *J Biol Chem* 280: 3613–3620, 2005.
158. **Liao L, Chen X, Wang S, Parlow AF, Xu J.** Steroid receptor coactivator 3 maintains circulating insulin-like growth factor I (IGF-I) by controlling IGF-binding protein 3 expression. *Mol Cell Biol* 28: 2460–2469, 2008.
159. **Liu B, Lee HY, Weinzimer SA, Powell DR, Clifford JL, Kurie JM, Cohen P.** Direct functional interactions between insulin-like growth factor-binding protein-3 and retinoid X receptor-alpha regulate transcriptional signaling and apoptosis. *J Biol Chem* 275: 33607–33613, 2000.
160. **Liu B, Lee KW, Anzo M, Zhang B, Zi X, Tao Y, Shiry L, Pollak M, Lin S, Cohen P.** Insulin-like growth factor-binding protein-3 inhibition of prostate cancer growth involves suppression of angiogenesis. *Oncogene* 26: 1811–1819, 2007.
161. **Liu B, Lee KW, Li H, Ma L, Lin GL, Chandraratna RA, Cohen P.** Combination therapy of insulin-like growth factor binding protein-3 and retinoid X receptor ligands synergize on prostate cancer cell apoptosis in vitro and in vivo. *Clin Cancer Res* 11: 4851–4856, 2005.
162. **Liu B, Weinzimer SA, Gibson TB, Mascarenhas D, Cohen P.** Type Ialpha collagen is an IGFBP-3 binding protein. *Growth Horm IGF Res* 13: 89–97, 2003.
163. **Lofqvist C, Chen J, Connor KM, Smith AC, Aderman CM, Liu N, Pintar JE, Ludwig T, Hellstrom A, Smith LE.** IGFBP3 suppresses retinopathy through suppression of oxygen-induced vessel loss and promotion of vascular regrowth. *Proc Natl Acad Sci USA* 104: 10589–10594, 2007.
164. **Loidl P, Loidl A, Puschendorf B, Grobner P.** RNA polymerase activity and template activity of chromatin after butyrate induced hyperacetylation of histones in Physarum. *Nucleic Acids Res* 12: 5405–5417, 1984.
165. **Lu XF, Jiang XG, Lu YB, Bai JH, Mao ZB.** Characterization of a novel positive transcription regulatory element that differentially regulates the insulin-like growth factor binding protein-3 (IGFBP-3) gene in senescent cells. *J Biol Chem* 280: 22606–22615, 2005.
166. **Mangasser-Stephan K, Gressner AM.** Molecular and functional aspects of latent transforming growth factor-beta binding protein: just a masking protein? *Cell Tissue Res* 297: 363–370, 1999.
167. **Mannhardt B, Weinzimer SA, Wagner M, Fiedler M, Cohen P, Jansen-Durr P, Zwerschke W.** Human papillomavirus type 16 E7 oncoprotein binds and inactivates growth-inhibitory insulin-like growth factor binding protein 3. *Mol Cell Biol* 20: 6483–6495, 2000.
168. **Martin JL, Baxter RC.** Transforming growth factor-beta stimulates production of insulin-like growth factor-binding protein-3 by human skin fibroblasts. *Endocrinology* 128: 1425–1433, 1991.
169. **Martin JL, Coverley JA, Pattison ST, Baxter RC.** Insulin-like growth factor-binding protein-3 production by MCF-7 breast cancer cells: stimulation by retinoic acid and cyclic adenosine monophosphate and differential effects of estradiol. *Endocrinology* 136: 1219–1226, 1995.
170. **Martin JL, Pattison SL.** Insulin-like growth factor binding protein-3 is regulated by dihydrotestosterone and stimulates deoxyribonucleic acid synthesis and cell proliferation in LNCaP prostate carcinoma cells. *Endocrinology* 141: 2401–2409, 2000.

171. **McCaig C, Perks CM, Holly JM.** Intrinsic actions of IGFBP-3 and IGFBP-5 on Hs578T breast cancer epithelial cells: inhibition or accentuation of attachment and survival is dependent upon the presence of fibronectin. *J Cell Sci* 115: 4293–4303, 2002.
172. **McCusker RH, Camacho-Hubner C, Bayne ML, Cascieri MA, Clemmons DR.** Insulin-like growth factor (IGF) binding to human fibroblast and glioblastoma cells: the modulating effect of cell released IGF binding proteins (IGFBPs). *J Cell Physiol* 144: 244–253, 1990.
173. **Megyesi K, Kahn CR, Roth J, Gorden P.** Circulating NSILA-s in man: Preliminary studies of stimuli in vivo and of binding to plasma components. *J Clin Endocrinol Metab* 41: 475–484, 1975.
174. **Meuli C, Zapf J, Froesch ER.** NSILA-carrier protein abolishes the action of nonsuppressible insulin-like activity (NSILA-S) on perfused rat heart. *Diabetologia* 14: 255–259, 1978.
175. **Miller BS, Khosravi MJ, Patterson MC, Conover CA.** The insulin-like growth factor system in children with congenital disorders of glycosylation. *Clin Endocrinol (Oxf)*, 2009.
176. **Mishra S, Raz A, Murphy LJ.** Insulin-like growth factor binding protein-3 interacts with autocrine motility factor/phosphoglucose isomerase (AMF/PGI) and inhibits the AMF/PGI function. *Cancer Res* 64: 2516–2522, 2004.
177. **Modric T, Silha JV, Shi Z, Gui Y, Suwanichkul A, Durham SK, Powell DR, Murphy LJ.** Phenotypic manifestations of insulin-like growth factor-binding protein-3 overexpression in transgenic mice. *Endocrinology* 142: 1958–1967, 2001.
178. **Murphy LJ, Molnar P, Lu X, Huang H.** Expression of human insulin-like growth factor-binding protein-3 in transgenic mice. *J Mol Endocrinol* 15: 293–303, 1995.
179. **Murphy LJ, Rajkumar K, Molnar P.** Phenotypic manifestations of insulin-like growth factor binding protein-1 (IGFBP-1) and IGFBP-3 overexpression in transgenic mice. *Prog Growth Factor Res* 6: 425–432, 1995.
180. **Muzumdar RH, Ma X, Fishman S, Yang X, Atzmon G, Vuguin P, Einstein FH, Hwang D, Cohen P, Barzilai N.** Central and opposing effects of IGF-I and IGF-binding protein-3 on systemic insulin action. *Diabetes* 55: 2788–2796, 2006.
181. **Nickerson T, Huynh H.** Vitamin D analogue EB1089-induced prostate regression is associated with increased gene expression of insulin-like growth factor binding proteins. *J Endocrinol* 160: 223–229, 1999.
182. **Ning Y, Schuller AG, Bradshaw S, Rotwein P, Ludwig T, Frystyk J, Pintar JE.** Diminished growth and enhanced glucose metabolism in triple knockout mice containing mutations of insulin-like growth factor binding protein-3, -4, and -5. *Mol Endocrinol* 20: 2173–2186, 2006.
183. **Oh Y, Gucev Z, Ng L, Muller HL, Rosenfeld RG.** Antiproliferative actions of insulin-like growth factor binding protein (IGFBP)-3 in human breast cancer cells. *Prog Growth Factor Res* 6: 503–512, 1995.
184. **Oh Y, Muller HL, Lamson G, Rosenfeld RG.** Insulin-like growth factor (IGF)-independent action of IGF-binding protein-3 in Hs578T human breast cancer cells. Cell surface binding and growth inhibition. *J Biol Chem* 268: 14964–14971, 1993.
185. **Oh Y, Muller HL, Ng L, Rosenfeld RG.** Transforming growth factor-beta-induced cell growth inhibition in human breast cancer cells is mediated through insulin-like growth factor-binding protein-3 action. *J Biol Chem* 270: 13589–13592, 1995.
186. **Oh Y, Muller HL, Pham H, Rosenfeld RG.** Demonstration of receptors for insulin-like growth factor binding protein-3 on Hs578T human breast cancer cells. *J Biol Chem* 268: 26045–26048, 1993.
187. **Ongeri EM, Verderame MF, Hammond JM.** The TATA binding protein associated factor 4b (TAF4b) mediates FSH stimulation of the IGFBP-3 promoter in cultured porcine ovarian granulosa cells. *Mol Cell Endocrinol* 278: 29–35, 2007.
188. **Oufattole M, Lin SW, Liu B, Mascarenhas D, Cohen P, Rodgers BD.** Ribonucleic acid polymerase II binding subunit 3 (Rpb3), a potential nuclear target of insulin-like growth factor binding protein-3. *Endocrinology* 147: 2138–2146, 2006.
189. **Pagano M, Benmaamar R.** When protein destruction runs amok, malignancy is on the loose. *Cancer Cell* 4: 251–256, 2003.
190. **Park R, Shepherd BS, Nishioka RS, Grau EG, Bern HA.** Effects of homologous pituitary hormone treatment on serum insulin-like growth-factor-binding proteins (IGFBPs) in hypophysectomized tilapia, Oreochromis mossambicus, with special reference to a novel 20-kDa IGFBP. *Gen Comp Endocrinol* 117: 404–412, 2000.
191. **Parker MI, de Haan JB, Gevers W.** DNA hypermethylation in sodium butyrate-treated WI-38 fibroblasts. *J Biol Chem* 261: 2786–2790, 1986.
192. **Peng L, Malloy PJ, Feldman D.** Identification of a functional vitamin D response element in the human insulin-like growth factor binding protein-3 promoter. *Mol Endocrinol* 18: 1109–1119, 2004.
193. **Peng L, Malloy PJ, Wang J, Feldman D.** Growth inhibitory concentrations of androgens up-regulate insulin-like growth factor binding protein-3 expression via an androgen response element in LNCaP human prostate cancer cells. *Endocrinology* 147: 4599–4607, 2006.
194. **Pratt SE, Pollak MN.** Estrogen and antiestrogen modulation of MCF7 human breast cancer cell proliferation is associated with specific alterations in accumulation of insulin-like growth factor-binding proteins in conditioned media. *Cancer Res* 53: 5193–5198, 1993.
195. **Radulescu RT.** Nuclear localization signal in insulin-like growth factor-binding protein type 3. *Trends Biochem Sci* 19: 278, 1994.
196. **Rajah R, Lee KW, Cohen P.** Insulin-like growth factor binding protein-3 mediates tumor necrosis factor-alpha-induced apoptosis: role of Bcl-2 phosphorylation. *Cell Growth Differ* 13: 163–171, 2002.
197. **Rajah R, Valentinis B, Cohen P.** Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta1 on programmed cell death through a p53- and IGF-independent mechanism. *J Biol Chem* 272: 12181–12188, 1997.
198. **Rehault S, Monget P, Mazerbourg S, Tremblay R, Gutman N, Gauthier F, Moreau T.** Insulin-like growth factor binding proteins (IGFBPs) as potential physiological substrates for human kallikreins hK2 and hK3. *Eur J Biochem* 268: 2960–2968, 2001.
199. **Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet SM, Egger M.** Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. *Lancet* 363: 1346–1353, 2004.
200. **Ricort JM, Binoux M.** Insulin-like growth factor-binding protein-3 activates a phosphotyrosine phosphatase. Effects on the insulin-like growth factor signaling pathway. *J Biol Chem* 277: 19448–19454, 2002.
201. **Ricort JM, Lombet A, Lassarre C, Binoux M.** Insulin-like growth factor binding protein-3 increases intracellular calcium concentrations in MCF-7 breast carcinoma cells. *FEBS Lett* 527: 293–297, 2002.
202. **Riggs MG, Whittaker RG, Neumann JR, Ingram VM.** n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. *Nature* 268: 462–464, 1977.
203. **Rinderknecht E, Humbel RE.** The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J Biol Chem* 253: 2769–2776, 1978.
204. **Roddam AW, Allen NE, Appleby P, Key TJ, Ferrucci L, Carter HB, Metter EJ, Chen C, Weiss NS, Fitzpatrick A, Hsing AW, Lacey JV Jr, Helzlsouer K, Rinaldi S, Riboli E, Kaaks R, Janssen JA, Wildhagen MF, Schroder FH, Platz EA, Pollak M, Giovannucci E, Schaefer C, Quesenberry CP Jr, Vogelman JH, Severi G, English DR, Giles GG, Stattin P, Hallmans G, Johansson M, Chan JM, Gann P, Oliver SE, Holly JM, Donovan J, Meyer F, Bairati I, Galan P.** Insulin-like growth factors, their binding proteins, and prostate cancer risk: analysis of individual patient data from 12 prospective studies. *Ann Intern Med* 149: 461–471, W483–468, 2008.
205. **Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG.** Caveolin, a protein component of caveolae membrane coats. *Cell* 68: 673–682, 1992.
206. **Rozen F, Zhang J, Pollak M.** Antiproliferative action of tumor necrosis factor-alpha on MCF-7 breastcancer cells is associated with increased insulin-like growth factor binding protein-3 accumulation. *Int J Oncol* 13: 865–869, 1998.
207. **Santer FR, Bacher N, Moser B, Morandell D, Ressler S, Firth SM, Spoden GA, Sergi C, Baxter RC, Jansen-Durr P, Zwerschke W.** Nuclear insulin-like growth factor binding protein-3 induces apoptosis and is targeted to ubiquitin/proteasome-dependent proteolysis. *Cancer Res* 66: 3024–3033, 2006.
208. **Santer FR, Moser B, Spoden GA, Jansen-Durr P, Zwerschke W.** Human papillomavirus type 16 E7 oncoprotein inhibits apoptosis mediated by nuclear insulin-like growth factor-binding protein-3 by enhancing its ubiquitin/proteasome-dependent degradation. *Carcinogenesis* 28: 2511–2520, 2007.
209. **Schedlich LJ, Graham LD, O'Han MK, Muthukaruppan A, Yan X, Firth SM, Baxter RC.** Molecular basis of the interaction between IGFBP-3 and retinoid X receptor: role in modulation of RAR-signaling. *Arch Biochem Biophys* 465: 359–369, 2007.
210. **Schedlich LJ, Le Page SL, Firth SM, Briggs LJ, Jans DA, Baxter RC.** Nuclear import of insulin-like growth factor-binding protein-3 and -5 is

mediated by the importin beta subunit. *J Biol Chem* 275: 23462–23470, 2000.

211. **Schedlich LJ, Nilsen T, John AP, Jans DA, Baxter RC.** Phosphorylation of insulin-like growth factor binding protein-3 by deoxyribonucleic acid-dependent protein kinase reduces ligand binding and enhances nuclear accumulation. *Endocrinology* 144: 1984–1993, 2003.
212. **Schedlich LJ, O'Han MK, Leong GM, Baxter RC.** Insulin-like growth factor binding protein-3 prevents retinoid receptor heterodimerization: implications for retinoic acid-sensitivity in human breast cancer cells. *Biochem Biophys Res Commun* 314: 83–88, 2004.
213. **Schedlich LJ, Young TF, Firth SM, Baxter RC.** Insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 share a common nuclear transport pathway in T47D human breast carcinoma cells. *J Biol Chem* 273: 18347–18352, 1998.
214. **Schwarze SR, DePrimo SE, Grabert LM, Fu VX, Brooks JD, Jarrard DF.** Novel pathways associated with bypassing cellular senescence in human prostate epithelial cells. *J Biol Chem* 277: 14877–14883, 2002.
215. **Shang Y, Baumrucker CR, Green MH.** Signal relay by retinoic acid receptors alpha and beta in the retinoic acid-induced expression of insulin-like growth factor-binding protein-3 in breast cancer cells. *J Biol Chem* 274: 18005–18010, 1999.
216. **Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, Campos D, Maoleekoonpiroj S, Smylie M, Martins R, van Kooten M, Dediu M, Findlay B, Tu D, Johnston D, Bezjak A, Clark G, Santabarbara P, Seymour L.** Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 353: 123–132, 2005.
217. **Shim ML, Levitt Katz LE, Davis J, Dotzler WC, Cohen P, Ferry RJ Jr.** Insulin-like growth factor binding protein-3 is a novel mediator of apoptosis in insulin-secreting cells. *Growth Horm IGF Res* 14: 216–225, 2004.
218. **Shon JK, Shon BH, Park IY, Lee SU, Fa L, Chang KY, Shin JH, Lee YI.** Hepatitis B virus-X protein recruits histone deacetylase 1 to repress insulin-like growth factor binding protein 3 transcription. *Virus Res*, 2008.
219. **Shukla S, Mishra A, Fu P, MacLennan GT, Resnick MI, Gupta S.** Up-regulation of insulin-like growth factor binding protein-3 by apigenin leads to growth inhibition and apoptosis of 22Rv1 xenograft in athymic nude mice. *FASEB J* 19: 2042–2044, 2005.
220. **Silha JV, Gui Y, Mishra S, Leckstrom A, Cohen P, Murphy LJ.** Overexpression of gly56/gly80/gly81-mutant insulin-like growth factor-binding protein-3 in transgenic mice. *Endocrinology* 146: 1523–1531, 2005.
221. **Silha JV, Gui Y, Murphy LJ.** Impaired glucose homeostasis in insulin-like growth factor-binding protein-3-transgenic mice. *Am J Physiol Endocrinol Metab* 283: E937–E945, 2002.
222. **Silha JV, Sheppard PC, Mishra S, Gui Y, Schwartz J, Dodd JG, Murphy LJ.** Insulin-like growth factor (IGF) binding protein-3 attenuates prostate tumor growth by IGF-dependent and IGF-independent mechanisms. *Endocrinology* 147: 2112–2121, 2006.
223. **Singh B, Charkowicz D, Mascarenhas D.** Insulin-like growth factor-independent effects mediated by a C-terminal metal-binding domain of insulin-like growth factor binding protein-3. *J Biol Chem* 279: 477–487, 2004.
224. **Singh RP, Dhanalakshmi S, Tyagi AK, Chan DC, Agarwal C, Agarwal R.** Dietary feeding of silibinin inhibits advance human prostate carcinoma growth in athymic nude mice and increases plasma insulin-like growth factor-binding protein-3 levels. *Cancer Res* 62: 3063–3069, 2002.
225. **Sloth Andersen A, Hertz Hansen P, Schaffer L, Kristensen C.** A new secreted insect protein belonging to the immunoglobulin superfamily binds insulin and related peptides and inhibits their activities. *J Biol Chem* 275: 16948–16953, 2000.
226. **Smart EJ, Graf GA, McNiven MA, Sessa WC, Engelman JA, Scherer PE, Okamoto T, Lisanti MP.** Caveolins, liquid-ordered domains, and signal transduction. *Mol Cell Biol* 19: 7289–7304, 1999.
227. **Smith GC, Jackson SP.** The DNA-dependent protein kinase. *Genes Dev* 13: 916–934, 1999.
228. **Soh J, Katsuyama M, Ushijima S, Mizutani Y, Kawauchi A, Yabe-Nishimura C, Miki T.** Localization of increased insulin-like growth factor binding protein-3 in diabetic rat penis: implications for erectile dysfunction. *Urology* 70: 1019–1023, 2007.
229. **Solomon SS, Poffenbarger PL, Hepp DK, Fenster LF, Ensinck JW, Williams RH.** Quantitation and partial characterization of nonsuppressible insulin-like activity in serum and tissue extracts of the rat. *Endocrinology* 81: 213–225, 1967.
230. **Song H, Beattie J, Campbell IW, Allan GJ.** Overlap of IGF- and heparin-binding sites in rat IGF-binding protein-5. *J Mol Endocrinol* 24: 43–51, 2000.
231. **Sordella R, Bell DW, Haber DA, Settleman J.** Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science* 305: 1163–1167, 2004.
232. **Spagnoli A, Torello M, Nagalla SR, Horton WA, Pattee P, Hwa V, Chiarelli F, Roberts CT Jr, and Rosenfeld RG.** Identification of STAT-1 as a molecular target of IGFBP-3 in the process of chondrogenesis. *J Biol Chem* 277: 18860–18867, 2002.
233. **Terashita K, Hashimoto Y, Niikura T, Tajima H, Yamagishi Y, Ishizaka M, Kawasumi M, Chiba T, Kanekura K, Yamada M, Nawa M, Kita Y, Aiso S, Nishimoto I.** Two serine residues distinctly regulate the rescue function of Humanin, an inhibiting factor of Alzheimer's disease-related neurotoxicity: functional potentiation by isomerization and dimerization. *J Neurochem* 85: 1521–1538, 2003.
234. **Tsubaki J, Hwa V, Twigg SM, Rosenfeld RG.** Differential activation of the IGF binding protein-3 promoter by butyrate in prostate cancer cells. *Endocrinology* 143: 1778–1788, 2002.
235. **Twigg SM, Kiefer MC, Zapf J, Baxter RC.** Insulin-like growth factor-binding protein 5 complexes with the acid-labile subunit. Role of the carboxyl-terminal domain. *J Biol Chem* 273: 28791–28798, 1998.
236. **Valentinis B, Bhala A, DeAngelis T, Baserga R, Cohen P.** The human insulin-like growth factor (IGF) binding protein-3 inhibits the growth of fibroblasts with a targeted disruption of the IGF-I receptor gene. *Mol Endocrinol* 9: 361–367, 1995.
237. **Vasylyeva TL, Chen X, Ferry RJ Jr.** Insulin-like growth factor binding protein-3 mediates cytokine-induced mesangial cell apoptosis. *Growth Horm IGF Res* 15: 207–214, 2005.
238. **Velayudhan BT, Govoni KE, Hoagland TA, Zinn SA.** Growth rate and changes of the somatotropic axis in beef cattle administered exogenous bovine somatotropin beginning at two hundred, two hundred fifty, and three hundred days of age. *J Anim Sci* 85: 2866–2872, 2007.
239. **Verrijdt G, Haelens A, Claessens F.** Selective DNA recognition by the androgen receptor as a mechanism for hormone-specific regulation of gene expression. *Mol Genet Metab* 78: 175–185, 2003.
240. **Vijayababu MR, Arunkumar A, Kanagaraj P, Arunakaran J.** Effects of quercetin on insulin-like growth factors (IGFs) and their binding protein-3 (IGFBP-3) secretion and induction of apoptosis in human prostate cancer cells. *J Carcinog* 5: 10, 2006.
241. **Vorwerk P, Yamanaka Y, Spagnoli A, Oh Y, Rosenfeld RG.** Insulin and IGF binding by IGFBP-3 fragments derived from proteolysis, baculovirus expression and normal human urine. *J Clin Endocrinol Metab* 83: 1392–1395, 1998.
242. **Walker GE, Wilson EM, Powell D, Oh Y.** Butyrate, a histone deacetylase inhibitor, activates the human IGF binding protein-3 promoter in breast cancer cells: molecular mechanism involves an Sp1/Sp3 multiprotein complex. *Endocrinology* 142: 3817–3827, 2001.
243. **Wang D, Nagpal ML, Shimasaki S, Ling N, Lin T.** Interleukin-1 induces insulin-like growth factor binding protein-3 gene expression and protein production by Leydig cells. *Endocrinology* 136: 4049–4055, 1995.
244. **Weinzimer SA, Gibson TB, Collett-Solberg PF, Khare A, Liu B, Cohen P.** Transferrin is an insulin-like growth factor-binding protein-3 binding protein. *J Clin Endocrinol Metab* 86: 1806–1813, 2001.
245. **Wiley A, Katsaros D, Chen H, Rigault de la Longrais IA, Beeghly A, Puopolo M, Singal R, Zhang Y, Amoako A, Zelterman D, Yu H.** Aberrant promoter methylation of multiple genes in malignant ovarian tumors and in ovarian tumors with low malignant potential. *Cancer* 107: 299–308, 2006.
246. **Wilson AJ, Arango D, Mariadason JM, Heerdt BG, Augenlicht LH.** TR3/Nur77 in colon cancer cell apoptosis. *Cancer Res* 63: 5401–5407, 2003.
247. **Wood WI, Cachianes G, Henzel WJ, Winslow GA, Spencer SA, Hellmiss R, Martin JL, Baxter RC.** Cloning and expression of the growth hormone-dependent insulin-like growth factor-binding protein. *Mol Endocrinol* 2: 1176–1185, 1988.
248. **Wraight CJ, Liepe IJ, White PJ, Hibbs AR, Werther GA.** Intranuclear localization of insulin-like growth factor binding protein-3 (IGFBP-3) during cell division in human keratinocytes. *J Invest Dermatol* 111: 239–242, 1998.

249. **Wraight CJ, Werther GA.** Insulin-like growth factor-I and epidermal growth factor regulate insulin-like growth factor binding protein-3 (IGFBP-3) in the human keratinocyte cell line HaCaT. *J Invest Dermatol* 105: 602–607, 1995.
250. **Wu C, Yao G, Zou M, Chen G, Wang M, Liu J, Wang J, Xu D.** N-Acetylgalactosaminyltransferase 14, a novel insulin-like growth factor binding protein-3 binding partner. *Biochem Biophys Res Commun* 357: 360–365, 2007.
251. **Xiao CY, Hubner S, Jans DA.** SV40 large tumor antigen nuclear import is regulated by the double-stranded DNA-dependent protein kinase site (serine 120) flanking the nuclear localization sequence. *J Biol Chem* 272: 22191–22198, 1997.
252. **Xu S, Cwyfan-Hughes SC, van der Stappen JW, Sansom J, Burton JL, Donnelly M, Holly JM.** Insulin-like growth factors (IGFs) and IGF-binding proteins in human skin interstitial fluid. *J Clin Endocrinol Metab* 80: 2940–2945, 1995.
253. **Yakar S, Rosen CJ, Bouxsein ML, Sun H, Mejia W, Kawashima Y, Wu Y, Emerton K, Williams V, Jepsen K, Schaffler MB, Majeska RJ, Gavrilova O, Gutierrez M, Hwang D, Pennisi P, Frystyk J, Boisclair Y, Pintar J, Jasper H, Domene H, Cohen P, Clemmons D, Leroith D.** Serum complexes of insulin-like growth factor-1 modulate skeletal integrity and carbohydrate metabolism. *FASEB J*, 2008.
254. **Yamamoto H, Murphy LJ.** Enzymatic conversion of IGF-I to des(1-3)IGF-I in rat serum and tissues: a further potential site of growth hormone regulation of IGF-I action. *J Endocrinol* 146: 141–148, 1995.
255. **Yamamoto H, Murphy LJ.** Generation of des-(1–3) insulin-like growth factor-I in serum by an acid protease. *Endocrinology* 135: 2432–2439, 1994.
256. **Yamanaka Y, Fowlkes JL, Wilson EM, Rosenfeld RG, Oh Y.** Characterization of insulin-like growth factor binding protein-3 (IGFBP-3) binding to human breast cancer cells: kinetics of IGFBP-3 binding and identification of receptor binding domain on the IGFBP-3 molecule. *Endocrinology* 140: 1319–1328, 1999.
257. **Yan X, Forbes BE, McNeil KA, Baxter RC, Firth SM.** Role of N- and C-terminal residues of insulin-like growth factor (IGF)-binding protein-3 in regulating IGF complex formation and receptor activation. *J Biol Chem* 279: 53232–53240, 2004.
258. **Yandell CA, Francis GL, Wheldrake JF, Upton Z.** Kangaroo IGF-II is structurally and functionally similar to the human [Ser29]-IGF-II variant. *J Endocrinol* 161: 445–453, 1999.
259. **Zapf J, Hauri C, Waldvogel M, Futo E, Hasler H, Binz K, Guler HP, Schmid C, Froesch ER.** Recombinant human insulin-like growth factor I induces its own specific carrier protein in hypophysectomized and diabetic rats. *Proc Natl Acad Sci USA* 86: 3813–3817, 1989.
260. **Zhang Y, Dawson MI, Mohammad R, Rishi AK, Farhana L, Feng KC, Leid M, Peterson V, Zhang XK, Edelstein M, Eilander D, Biggar S, Wall N, Reichert U, Fontana JA.** Induction of apoptosis of human B-CLL and ALL cells by a novel retinoid and its nonretinoidal analog. *Blood* 100: 2917–2925, 2002.
261. **Zhao Y, Yin P, Bach LA, Duan C.** Several acidic amino acids in the N-domain of insulin-like growth factor-binding protein-5 are important for its transactivation activity. *J Biol Chem* 281: 14184–14191, 2006.
262. **Zhou Y, Mohan S, Linkhart TA, Baylink DJ, Strong DD.** Retinoic acid regulates insulin-like growth factor-binding protein expression in human osteoblast cells. *Endocrinology* 137: 975–983, 1996.
263. **Zhu J, Zhou W, Jiang J, Chen X.** Identification of a novel p53 functional domain that is necessary for mediating apoptosis. *J Biol Chem* 273: 13030–13036, 1998.
264. **Zimmermann EM, Li L, Hoyt EC, Pucilowska JB, Lichtman S, Lund PK.** Cell-specific localization of insulin-like growth factor binding protein mRNAs in rat liver. *Am J Physiol Gastrointest Liver Physiol* 278: G447–G457, 2000.
265. **Zingg AE, Froesch ER.** Effects of partially purified preparations with nonsuppressible insulin-like activity (NSILA-S) on sulfate incorporation into rat and chicken cartilage. *Diabetologia* 9: 472–476, 1973.

Nuclear export and mitochondrial and endoplasmic reticulum localization of IGF-binding protein 3 regulate its apoptotic properties

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Abstract

Tumor suppression by IGF-binding protein 3 (IGFBP3) may occur in an IGF-independent manner, in addition to its role as a regulator of IGF bioavailability. After secretion, IGFBP3 is internalized, rapidly localized to the nucleus, and is later detected in the cytoplasm. We identified a putative nuclear export sequence (NES) in IGFBP3 between amino acids 217 and 228, analogous to the leucine-rich NES sequence of p53 and HIV Rev. Mutation of the NES prevents nucleocytoplasmic shuttling of IGFBP3 and blocks its ability to induce apoptosis. Targeting of IGFBP3 to the mitochondria and endoplasmic reticulum (ER) was confirmed by co-localization with organelle markers using fluorescence confocal microscopy and subcellular fractionation. Mitochondrial targeting was also demonstrated *in vivo* in IGFBP3-treated prostate cancer xenografts. These results show that IGFBP3 shuttles from the nucleus to the mitochondria and ER, and that nuclear export is essential for its effects on prostate cancer apoptosis.

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Introduction

Insulin-like growth factor-binding protein 3 (IGFBP3) exerts its tumor suppressive properties via IGF-dependent and -independent mechanisms (for review see Firth & Baxter (2002)). IGFBP3 has been historically described as a modulator of cell growth through its IGF-dependent effects, specifically the transport of IGF1 to its receptor resulting in potentiation of IGF1 action, or sequestration of IGF1 from its cognate receptor, inhibiting its activity. Sequestering IGFs from signaling via their cognate receptor is an important property of IGFBP3 that could be explored in the therapy of cancer. However, IGFBP3 is now widely accepted to have additional IGF-independent properties (including apoptosis induction) that identify it as a unique peptide for cancer therapy.

Several advances in the field have enabled researchers to demonstrate evidence for IGF-independent effects of IGFBP3 using various strategies: 1) IGFBP3 mutants that do not bind to IGF1 (Buckway *et al*. 2001, Hong *et al*. 2002, Yan *et al*. 2004) or IGF1 analogs with reduced affinity for IGFBP3 (long R3-IGF1, GroPep, Australia, and des-(1–3)-IGF1; Yamamoto & Murphy 1994, 1995); 2) biologically active IGFBP3 fragments with total and partial loss of IGF affinity respectively (Lalou *et al*. 1996); 3) transfection of an IGFBP3 overexpression vector into cells lacking the type 1 IGF receptor (Valentinis *et al*. 1995, Rajah *et al*. 1997); and 4) use of IGF1 negative cell lines (breast cancer cells and chondrocytes; Gucev *et al*. 1996, Spagnoli *et al*. 2002).

Transgenic mice overexpressing either IGFBP3 or the non-IGF-binding mutant GGG-IGFBP3 mated with a mouse model of prostate cancer demonstrated convincingly that a) IGFBP3 potently inhibits prostate cancer growth and progression, and that b) these effects are mediated by both IGF-dependent and IGF-independent mechanisms (Silha *et al*. 2006). Together, *in vitro* and *in vivo* studies provide convincing evidence supporting IGF-independent actions of IGFBP3.

After secretion, IGFBP3 is internalized via endocytic mechanisms and localizes to the nucleus

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(Schedlich *et al*. 1998, 2000, Lee *et al*. 2004). This transport is mediated by importin-β and a C-terminal nuclear localization sequence (NLS; Schedlich *et al*. 2000). In addition, phosphorylation of IGFBP3 by DNA-dependent protein kinase enhances nuclear accumulation and its apoptotic activities (Cobb *et al*. 2006).

We previously proposed a role for IGFBP3 in the nucleo-mitochondrial translocation of the nuclear receptors RXRα and Nur77 to initiate the intrinsic apoptotic pathway and caspase activation, as well as demonstrating that IGFBP3 and Nur77 associate in the cytoplasmic compartment of 22RV1 cells (Lee *et al*. 2005, 2007). Importantly, the nuclear transcription factors p53, Nur77, and RXRα have been shown to be actively transported out of the nucleus to mitochondria dependent on leucine-rich nuclear export sequences (NES; Stommel *et al*. 1999, Cao *et al*. 2004, Moll *et al*. 2006).

For this study, we identified sequence elements in IGFBP3 that are required for nuclear export and mitochondrial localization. We found that nuclear export is required for IGFBP3-induced apoptosis, and that exogenously administered IGFBP3 localizes to mitochondria in 22RV1 prostate cancer xenografts. These findings suggest that IGFBP3 may be regulated by trafficking between nuclear and extranuclear targets, and that these extranuclear targets mediate its apoptotic effects in prostate cancer cells.

Materials and methods

Materials

Insmed (Glen Allen, VA, USA) provided recombinant human IGFBP3. Commercial antibodies included anti-human IGFBP3 from DSL (Webster, TX, USA) and R&D systems (Minneapolis, MN, USA); anti-human PDI (for ER identification) was from Stressgen (Ann Arbor, MI, USA); anti-human ACSL4 (for MAM identification) and anti-human RXRα was from Santa Cruz Biotechnologies (Santa Cruz, CA, USA); and control goat IgG from Santa Cruz Biotechnologies. Anti-human Bcl-2 and Hsp60 were from Abcam (Cambridge, MA, USA). Nur77 antibody was in-house generated and previously described (Lee *et al*. 2005). DNA PKcs antibody was from Kamiya Biochemical Company (Seattle, WA, USA). SDS-PAGE reagents, Tween, and fat-free milk were purchased from Bio-Rad. ECL reagents were from Amersham. All other chemicals were from Sigma–Aldrich.

Cell culture

22RV1 cells were maintained in RPMI 1840 medium containing 10% FCS (Life Technologies), 100 units of penicillin/ml, and 100 units of streptomycin/ml in a humidified environment with 5% CO_2. Fibroblasts from an IGF1 receptor knockout and corresponding wild-type (WT) mouse were a kind gift from Dr Renato Baserga (Sell *et al*. 1993, Valentinis *et al*. 1995). These cells are designated receptor-negative (R−) embryonic fibroblast cells (MEFs). The R-cells were maintained in DMEM containing 10% fetal bovine serum and geneticin (G418). All cells were used before passage 6.

Site-directed mutagenesis

Mutant NES IGFBP3 protein was constructed by conversion of two leucine residues – at positions 224 and 227 to alanines by second-round PCR overlap mutagenesis. Stratagene's QuikChange Primer Design Program at www.stratagene.com/qcprimerdesign was used for designing primers to introduce the site-directed mutations. Primers GGAAGACACACTGAATCACGCGAAGTTCGCCAATGTGCTGAGTCCCAGG and CCTGGGACTCAGCACATTGGCGAACTTCGCGTGATTCAGTGTGTCTTCC were used to generate the ^{224}L to A and ^{227}L to A double mutant. Underlined are the nucleotides introducing the mutations.

For subcloning into the pFLAG CMV vector (Sigma) and C-terminal FLAG sequence, fusion mutants were amplified with engineered EcoRI and KpnI restriction sites to the sense and antisense primers respectively: GCTGAATTCCCACCATGCAGCGGGCGCGACCCACG and ATCGGTACCCTACTTGTCATCGTCGTCCTTGTAATCCTTGCTCTGCATGCTGTAGCAGTGC. Mutants were confirmed by sequencing.

Transient transfections and cell death ELISA

Cells (2×10^4) were seeded in 96-well culture plates. Reagents were appropriately scaled up to six-well plates for transfections that were followed by mitochondrial isolation and subsequent western immunoblotting. Transfections were done with LipofectAMINE:PLUS Reagent as directed by the manufacturer (Invitrogen). Typically, 50 ng β-galactosidase expression vector (pSV-β-Gal, Promega) and 50 ng expression vector were mixed with carrier DNA to give 0.2 μg total DNA per well. After 24–48 h of transfection, photometric Cell Death ELISA (Roche Applied Science) was

performed according to the manufacturer's instructions to quantify histone-associated DNA fragments (mono- and oligonucleosomes) generated by apoptotic cells.

Co-immunoprecipitation and western immunoblots

22RV1 nuclear or cytoplasmic extracts were immunoprecipitated with control IgG, anti-IGFBP3, or anti-RXRα antibodies. Briefly, 250 μl of protein A-agarose were incubated overnight at 4 °C with 5 μl of anti-human IGFBP3 antibodies. About 125 μl of each antibody-treated protein A-agarose were added to 10 μg protein extract and incubated for 3 h at 4 °C with shaking. Immunoprecipitated proteins were pelleted by centrifugation and washed three times with 500 μl IP wash buffer (1×PBS($Ca^{2+}Mg^{2+}$free), 0.05% NP40, 0.02% NaN_3). Two hundred microliters sample buffer was added to each sample and vortexed vigorously. Samples were boiled and vortexed again to release protein–antibody complexes from the protein A-agarose. The protein A-agarose was then separated from the immunoprecipitated complexes by centrifugation. The supernatants were saved, and the immunoprecipitated proteins were separated by nonreducing SDS-PAGE (8%) at constant voltage overnight, then transferred to PVDF for 4 h at 170 mA. The nitrocellulose was immersed in blocking solution (5% nonfat milk/PBS) for 45 min, washed with TST wash buffer (0.15 M NaCl, 0.01 M Tris base, 0.3% Tween 20, pH 7.4), 0.1% Tween, and incubated with primary anti-human IGFBP3 antibody (1:4000) for 2 h. After washing off any unbound antibodies, the PVDF was incubated with a secondary antibody (1:10 000) for 1 h. The membrane was washed four times with TST wash buffer. Bands were visualized using the peroxidase-linked enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech). Experiments were repeated three times.

Subcellular fractionation procedures

Nu-CLEAR Protein Extraction Kit was from Sigma–Aldrich, and isolation was followed according to the manufacturer's protocol.

Isolation of mitochondria, endoplasmic reticulum, and mitochondria associated membrane fractions

Membrane-associated fractions were isolated according to the published protocols (Bozidis *et al*. 2007). This procedure combines differential centrifugation (for separation of the postnuclear supernatant from nuclei and cellular debris at low ***g*** forces) and a self-generating Percoll gradient centrifugation.

Immunoflourescence confocal microscopy

For overexpression staining, 30 000 cells per chamber were plated, and 200 ng plasmid was transfected using Lipofectamine 2000 (Invitrogen) and cells were used for immunohistochemistry 48 h after transfection. Cells were stained live with MitoTracker Deep Red 633 FM (Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were fixed for 10 min in 4% paraformaldehyde in PBS and permeabilized in 0.2% Tween 20. Incubations with primary antibody and secondary reagent were done for 1 h at room temperature. For the FLAG-tagged IGFBP3 construct, anti-FLAG antibody rabbit polyclonal (Cell Signaling, Danvers, MA, USA) was used at 400× dilution, followed by Alexa Fluor 488 Goat Anti-Rabbit conjugate (Molecular Probes) at 1:400, i.e. 5 μg/ml final concentration. Counterstaining of nuclei was done with DAPI, and cells were mounted in ProLong Gold antifade reagent (Molecular Probes). Acquisitions were done using Leica DM IRE2 Microscope and Leica Confocal Software. Endogenous IGFBP3 was detected with anti-human IGFBP3 goat polyclonal antibody (R&D), followed by Alexa Fluor 488 (Molecular Probes). Endoplasmic reticulum (ER) was labeled with anti-human PDI mouse monoclonal antibody (Stressgen), followed by Alexa Fluor 633 rabbit anti-mouse IgG (Molecular Probes). For ER staining, samples were stained according to the manufacturer's references (i.e. only methanol fixation/permeabilization for 20 min at −20 °C was carried without fixation in paraformaldehyde).

Tumor xenografts

22RV1 xenograft tumors were generated by injection of 1×10^6 cells in 200 μl mixed at a 1:1 dilution with Matrigel in the right flank of male SCID mice. Tumors were established for 2 weeks before the start of treatment. Ten SCID mice with 22RV1 tumors were treated twice per day with saline or IGFBP3 (40 mg/kg per day) given by divided i.p. injections. The mice were killed after 3 days. Tumors were harvested, and immediately processed with Mitochondria Isolation Kit (animal tissue specific, Sigma). Mitochondrial proteins were resolved by SDS-PAGE and immunoblotted with IGFBP3 antibody (DSL).

Statistical analysis

All experiments were repeated at least three times. Means ± S.D. are shown. Statistical analyses were performed using ANOVA utilizing InStat (GraphPad, San Diego, CA, USA). Differences were considered statistically significant when $P < 0.005$, denoted by **.

Results

Rapid IGF-independent nuclear localization of IGFBP3 and cytoplasmic export

Nuclear localization of IGFBP3 is a well-described phenomenon and has been demonstrated in a variety of cellular models (Jaques *et al*. 1997, Li *et al*. 1997, Wraight *et al*. 1998, Liu *et al*. 2000, Sun *et al*. 2008). IGFBP3 possesses a consensus bipartite NLS (Radulescu 1994), and nuclear transport is facilitated by importin-β factor (Schedlich *et al*. 2000). We have begun to characterize the intracellular trafficking of IGFBP3 and its relation to biological function. We have also recently published mechanisms involved in the internalization of IGFBP3 after secretion (Lee *et al*. 2004). After internalization by endocytic pathways, IGFBP3 is first targeted to the nucleus (Fig. 1A). About 500 ng of rhIGFBP3 was added to 22RV1 CaP cells for 15 min. Subcellular fractions were isolated, and IGFBP3 was immunoprecipitated with goat polyclonal anti-IGFBP3 antibody. Immunoprecipitation of treated cell lysate with control IgG is also shown on the right. Proteins were resolved by SDS-PAGE, and IGFBP3 was identified by immunoblotting with mouse monoclonal anti-IGFBP3 antibody. Within 15 min of addition of IGFBP3, we were able to detect IGFBP3 in the nucleus, suggesting that this is the initial destination of internalized IGFBP3. To expand the time course and explore the IGF-independent intracellular trafficking of IGFBP3, we utilized IGF R− MEFs derived from an IGF1R knockout mouse. These cells have been shown previously to neither bind nor respond to IGFs (Sell *et al*. 1993). R-MEFs were pulsed with 500 ng of IGFBP3, and its subcellular localization was followed over a 180 min time course (Fig. 1B). To demonstrate the purity of the fractionation, expression of mitochondria-specific protein Hsp60 and nuclear-specific protein poly(ADP-ribosyl) polymerase (PARP) is shown. Again, the primary destination of IGFBP3 in 15 min is the nucleus, after which levels in the cytoplasm begin to increase, consistent with an active export mechanism.

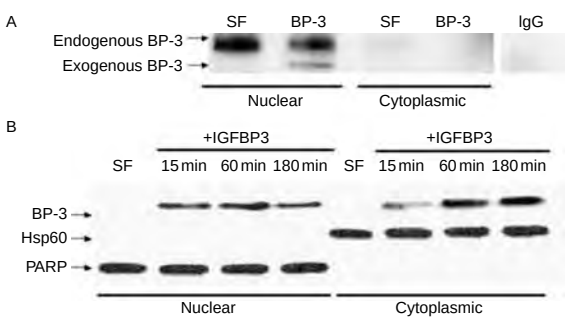

Figure 1 IGFBP3 enters the nucleus rapidly and subsequently is exported to the cytoplasm. (A) 22RV1 CaP cells treated with IGFBP3 for 15 min, and subcellular fractions were immunoprecipitated and immunoblotted with IGFBP3 antibody. (B) Time course of IGFBP3 subcellular localization in R-MEF cells, Hsp60, and PARP were used to assess purity of the subcellular fractions. SF, serum-free control.

A highly conserved NES lies within the C-terminal domain of IGFBP3

Because of the preliminary data in Fig. 1 showing the shuttling of IGFBP3 from the nucleus to the cytoplasm, we examined the primary amino acid sequence of IGFBP3 to determine whether it contains a leucine-rich sequence of conserved spacing and hydrophobicity, which fits the criteria established for an NES (Bogerd *et al*. 1996, Kim *et al*. 1996). We observed that the C-terminal residues between amino acids 190 and 201 conform to this motif, as indicated by their similarity to other known NESs such as HIV Rev and p53 (Table 1; Fischer *et al*. 1995, Stommel *et al*. 1999). In addition, alignment of this sequence across other members of the IGFBP family is shown. This sequence is highly conserved in widely divergent species. In addition to its role as a nuclear transcription factor, p53 has a confirmed pro-apoptotic role at the mitochondria (Mihara *et al*. 2003). This putative sequence suggests extranuclear trafficking of IGFBP3 and the possibility of a mitochondrial function for IGFBP3.

Table 1 A nuclear export sequence (NES) in insulin-like growth factor-binding protein 3 (IGFBP3)

p53	340	**MF**REL**L**NE**AL**EL**K**	351
HIV rev	72	**L**P-P**L**-ER**LTL**D	84
BP-3	217	**M**EDT**L**-NH**LKF**L	227
BP-5	196	**M**EAS**L**-QEL**KA**S	206
BP-4	178	**L**HR**AL**-ER**LAA**S	188
BP-2	234	**L**DQ**VL**-ERI**STM**	244
BP-6	167	**L**DS**VL**-QQ**L**QTE	177
BP-1	194	SGEE**I**-SK**FYL**P	204

This region was identified in the C-terminal region of IGFBP3 between AA 217 and 228 due to similarity to established NES in regions of conserved spacing and hydrophobicity in p53 and HIV Rev proteins. Analogous regions of other members of the IGFBP family are shown in descending order of similarity. Hydrophobic amino acids are highlighted in bold.

We constructed a mutant NES IGFBP3:FLAG (C-terminal) fusion consisting of leucine to alanine conversions at residues 197 and 200, since analogous mutations in other NES-containing proteins have been reported to prevent nuclear export (Bogerd *et al*. 1996, Kim *et al*. 1996, Stommel *et al*. 1999). WT or mutant NES:FLAG constructs were cloned in expression vectors, verified by sequencing, and transiently transfected to assess subcellular localization.

Western immunoblotting revealed that anti-IGFBP3 antibody recognized the mutant NES (Fig. 2A). Fractionation of transfected 22RV1 prostate cancer cells into nuclear and cytoplasmic fractions revealed that whereas WT IGFBP3 had equal distribution between nuclear and cytoplasmic fractions 48 h after transfection, IGFBP3 NES mutant displayed increased expression in nuclear versus cytoplasmic fractions compared with WT IGFBP3 (Fig. 2B). Densitometric quantitation of the IGFBP3 bands revealed a dramatic increase in nuclear retention of IGFBP3 with concomitant decrease in cytoplasmic localization. Immunoblotting with DNA PKcs and with Hsp60 were used to assess purity of the nuclear and cytoplasmic fractions respectively.

Analysis by indirect immunofluorescent confocal microscopy correlated with subcellular fractionation for IGFBP3 localization with the mutant NES, showing increased accumulation in the nucleus (Fig. 2C).

Impaired nuclear export of IGFBP3 retains RXRα/Nur77 heterodimers in the nucleus and abolishes the apoptotic actions of IGFBP3

We have previously described nucleo-mitochondrial translocation of nuclear receptor RXRα/Nur77 heterodimers to activate the mitochondrial pathway in apoptosis induction by IGFBP3 (Lee *et al*. 2005, 2007). We studied the effect of overexpressing mNES IGFBP3 on the subcellular localization of RXRα/Nur77. Mutation of the IGFBP3 NES did not impair the interaction with RXRα or Nur77 (data not shown). mNES as well as WT IGFBP3 expression vector was transiently transfected in 22RV1 CaP cells, and cell lysates were fractionated into nuclear and cytoplasmic fractions. Fractions were then immunoprecipitated with anti-RXRα antibody and immunoblotted with anti-Nur77. Overexpression of mNES IGFBP3 results in a marked increase in nuclear retention of RXRα/Nur77 (Fig. 3A), impairing nuclear export of important mediators of the apoptotic action of IGFBP3.

To directly examine the functional relationship between the localization of IGFBP3 and its effects on apoptosis, we transiently transfected 22RV1 prostate cancer cells with expression vector, WT IGFBP3, and mNES IGFBP3 (Fig. 3B). Transfection of WT IGFBP3 in 22RV1 cells increased oligonucleosomal fragments, as measured by a cell death ELISA, by 56% compared with empty vector transfection control. Retaining IGFBP3 in the nucleus by mutating the NES completely inhibited the ability of IGFBP3 to induce apoptosis. These studies confirm the integral role of extranuclear trafficking of IGFBP3 via its NES for its apoptotic properties.

Association of IGFBP3 with mitochondria and ER *in vitro*

In an attempt to obtain more detailed information on IGFBP3 subcellular protein localization of endogenous IGFBP3 in 22RV1 prostate cancer cells, we utilized a subcellular fractionation that combines differential and Percoll gradient centrifugations, as this is the preferred method for higher purity fractions (Bozidis *et al*. 2007). The internal membranes were segregated into mitochondria (Fig. 4A, lane 1), mitochondria-associated membrane (MAM; lane 2), ER (lane 3), and a pellet from the MAM fraction that was collected at low ***g*** centrifugation, representing an intermediate zone between mitochondria and MAM fraction (lane 4). The MAM fraction, a subdomain of the ER which consists of membrane tubules that provide direct physical contact between the ER and mitochondria, was fractionated to high purity (Vance 1990). The presence of IGFBP3 in the various membrane fractions was assessed by immunoblotting. The relative purity of the fractions was assessed by the presence of specific marker antibodies (Hsp60–mitochondria; PDI–ER; ACSL4–MAM). Under baseline conditions *in vitro*, IGFBP3 localizes to the mitochondria (lane 1), and is even more abundantly represented in the ER and MAM membrane fractions (lanes 2 and 3 respectively). Bcl-2 expression is detected in all membrane fractions, as previously described (Lithgow *et al*. 1994). RXRα was detected most prominently in the mitochondria fraction, as well as a faint presence in the MAM. The presence of RXRα in lane 4 may represent mitochondria in this transitional layer. In addition, Nur77, another binding partner of IGFBP3, was identified in the MAM and ER, with faint presence in the mitochondrial fraction. Mitochondrial localization of RXRα and Nur77 has been described previously (Cao *et al*. 2004). Immunoflourescence studies revealed co-localization of endogenous IGFBP3 with Mitotracker and PDI, an ER marker, consistent with the subcellular fractionation studies (Fig. 4B).

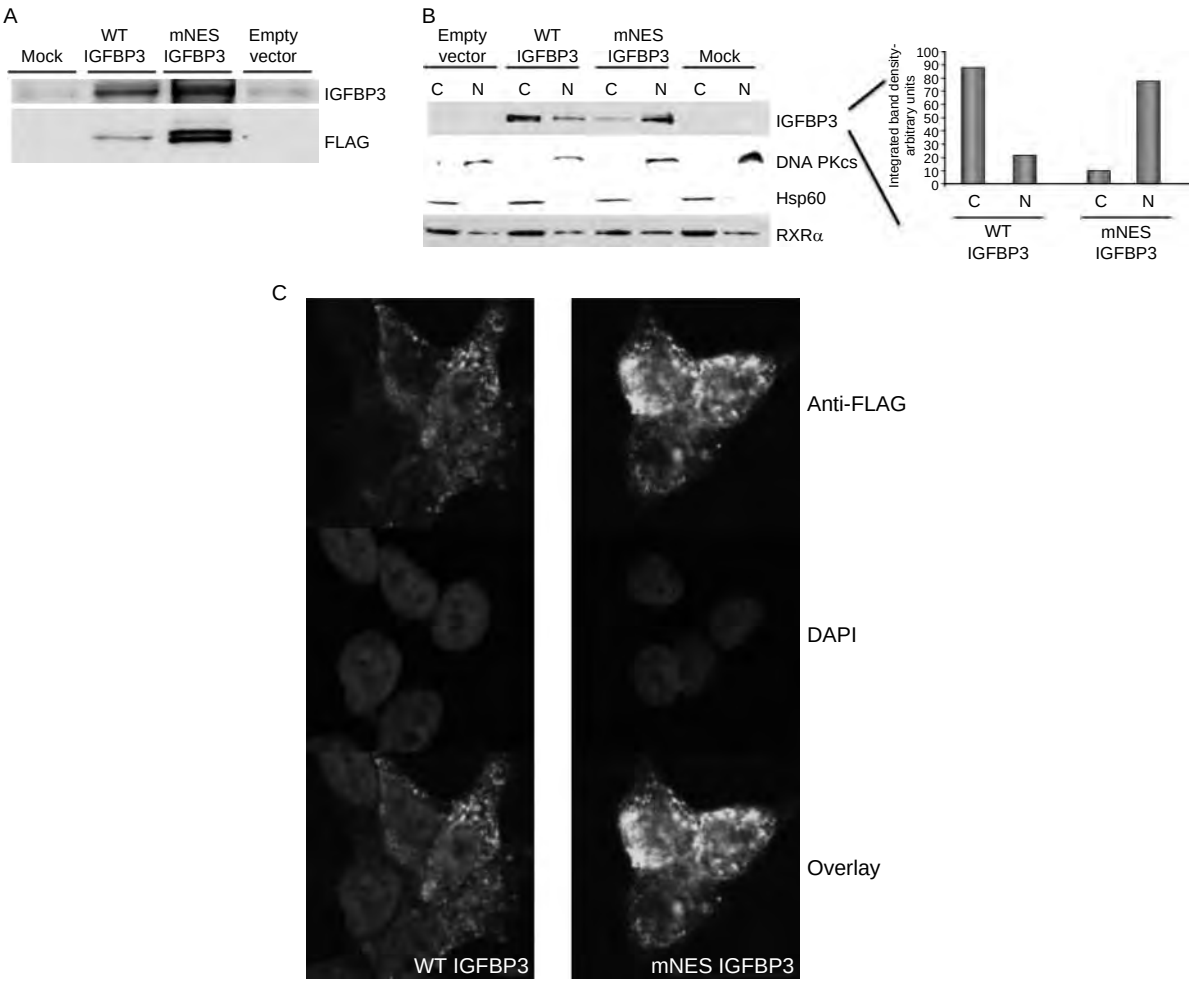

Figure 2 Subcellular localization of IGFBP3 mutants. (A) Recognition of mutant IGFBP3 by IGFBP3 antibody. (B) mNES IGFBP3 accumulates in the nucleus. 22RV1 cells were transfected with WT and mutant IGFBP3 expression vectors, and fractionated into cytoplasmic and nuclear fractions 48 h after transfection. IGFBP3 NES mutant shows increased amounts in nuclear versus cytoplasmic fractions compared to WT IGFBP3. Quantitation of densitometry for IGFBP3 shown on right. C, cytoplasmic fraction; N, nuclear fraction. (C) Immunofluouresence of transfected WT and mutant IGFBP3 in 22RV1 prostate cancer cells. Green, anti-FLAG (IGFBP3); blue, DAPI (nuclear staining).

Mitochondrial targeting of IGFBP3 *in vivo*

To determine whether administered IGFBP3 is targeted to mitochondria *in vivo*, we administered saline or recombinant human IGFBP3 (Insmed, Inc.) at 40 mg/kg i.p. divided twice a day for 3 days to male SCID mice carrying 22RV1 prostate cancer tumors. Over this short-time period, tumor size did not decrease (data not shown) as we have described for a longer incubation (Liu *et al*. 2007), and as others have described in a transgenic model utilizing a non-IGF-binding mutant (Cohen 2006, Silha *et al*. 2006). Mitochondria were isolated utilizing the Sigma animal tissue mitochondrial isolation kit, and associated proteins were resolved by SDS-PAGE (Fig. 5). In three of four tumors examined, IGFBP3 was found to be associated with mitochondria. Hsp60 and PARP were used to assess purity of the mitochondrial fraction.

Discussion

In the present study, we have expanded on previous structural elements that regulate the intracellular localization of IGFBP3, namely a C-terminal NLS (Schedlich *et al*. 2000). In the nucleus, IGFBP3 may interact with the nuclear receptors RXRα and RAR to modulate DNA transcription (Liu *et al*. 2000, Schedlich *et al*. 2004), not unlike related binding protein IGFBP5 which also localizes to cell nuclei and

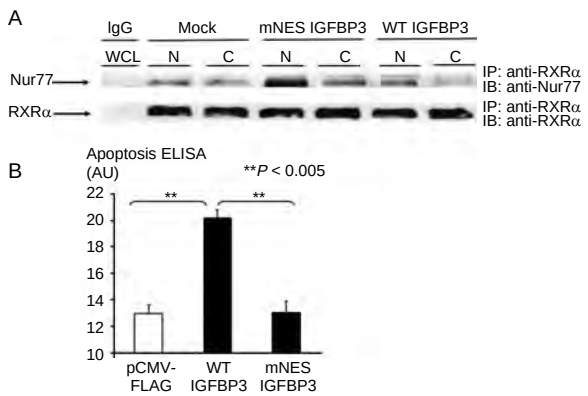

Figure 3 Nuclear export is required for the apoptotic actions of IGFBP3. (A) Overexpression of mNES IGFBP3 in 22RV1 cells induces nuclear retention of RXRα/Nur77 protein complexes compared to WT IGFBP3 overexpression. Mock transfection and co-immunoprecipitation with control IgG are shown on the left. After overexpression, cell lysates were fractionated and immunoprecipitation was performed as indicated. (B) Transfection of wild-type IGFBP3 in 22RV1 cells increased oligonucleosomal fragments by 56% compared to empty vector transfection control. Retaining IGFBP3 in the nucleus by mutating the NES blocked the ability of IGFBP3 to induce apoptosis.

interacts with RXRα, RAR, and the vitamin D receptor (Schedlich *et al*. 2004, 2007). In addition, nuclear ubiquitination of IGFBP3 on lysine residues with subsequent degradation has been described (Santer *et al*. 2006). We have demonstrated cytoplasmic interaction of endogenous IGFBP3 with the nuclear receptor Nur77 (Lee *et al*. 2007). Upon addition of exogenous IGFBP3, RXRα/Nur77 heterodimers translocate to mitochondria and activate the intrinsic apoptotic pathway (Lee *et al*. 2005).

Upon internalization by the cell, IGFBP3 is rapidly endocytosed to the nucleus and is actively transported to the cytoplasm, since mutations in the NES cause nuclear accumulation of IGFBP3. We also describe the mitochondrial localization of endogenous IGFBP3, as evidenced by subcellular fractionation and immunofluouresence confocal microscopy. Significantly, we have recently published that IGFBP3 and BAX interaction at the mitochondria activates testicular germ cell apoptosis (Jia *et al*. 2009). IGFBP1 has been reported to be up-regulated in response to p53 activation and antagonizes p53 mitochondrial accumulation secondary to BAK inactivation at mitochondria in hepatocytes (Leu & George 2007). However, IGFBP1 has not been described in the nucleus and is most dissimilar to the other IGFBPs in the leucine-rich hydrophobicity characteristic of NES (Table 1). This, combined with the fact that sequence analysis by subcellular localization bioinformatics programs does not indicate a mitochondrial targeting sequence in IGFBP1 (data not shown), means that intracellular trafficking similar to what we describe for IGFBP3 has not been previously demonstrated.

We also describe the initial description of endogenous localization for IGFBP3 and its binding partner the nuclear receptor Nur77 in the ER. Although we cannot rule out that IGFBP3 presence in the ER is not evidence of *de novo* ribosomal synthesis, complete absence of other probed proteins (e.g. RXRα and Hsp60) would argue against constitutive background translation. Signaling pathways emanating from the ER are involved in apoptosis initiated by stimuli as diverse as ER stress, oncogene expression, death

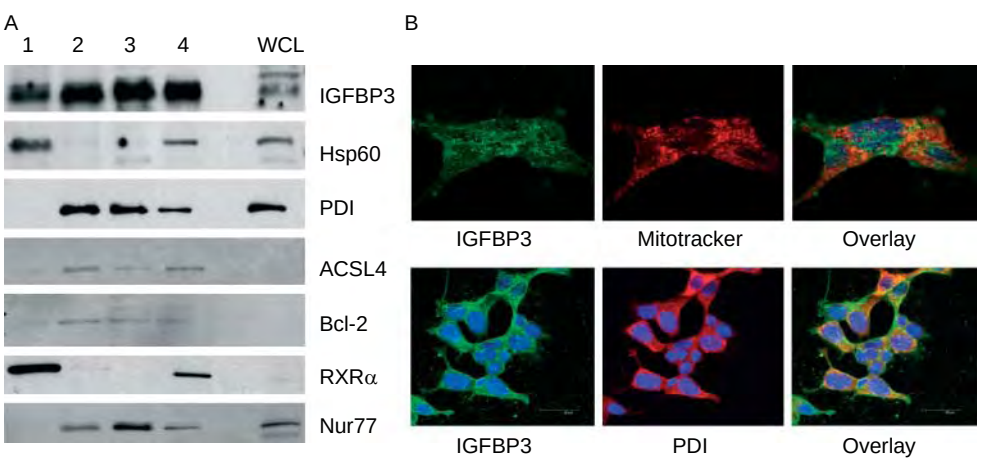

Figure 4 Endogenous mitochondria and ER localization of IGFBP3. (A) 22RV1 prostate cancer cells were fractionated into lane 1, mitochondrial fraction; lane 2, mitochondria-associated membranes (MAM); lane 3, endoplasmic reticulum (ER); and lane 4, pellet from MAM. (b) Co-localization (yellow) of IGFBP3 (green) with mitochondrial (Mitotracker, red) and ER markers (PDI, red) by immunofluouresence confocal microscopy.

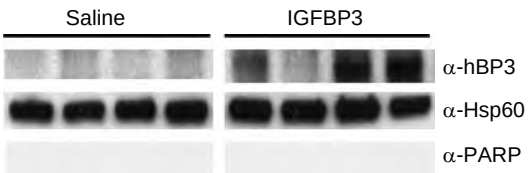

Figure 5 Prostate cancer xenografts internalize recombinant human IGFBP3 (rhIGFBP3), which localizes to mitochondria *in vivo*. 22RV1 CaP xenografts on SCID male mice were treated with IGFBP3, mitochondria were isolated, and immunoblotted for IGFBP3.

receptor ligation, and oxidative stress (Malhotra & Kaufman 2007, Heath-Engel *et al*. 2008). Multiple pathways may be involved in ER stress-initiated apoptosis, including crosstalk with the mitochondria (Hetz 2007). IGFBP3 has been associated with a rapid increase in intracellular Ca^{2+} concentrations (Ricort *et al*. 2002). The authors proposed the activation of a G-coupled protein by IGFBP3 as this was a pertussis toxin-sensitive pathway. However, radio-iodinated IGFBP3 was utilized, and in our experience, it does not internalize into cells (data not shown). As the ER lumen represents that the major site of intracellular Ca^{2+} storage and release has been implicated in ER:mitochondria apoptosis cross-talk (Scorrano *et al*. 2003), it is intriguing to speculate that IGFBP3 ER localization may have a role in this process. The observation of IGFBP3 localization to the ER and mitochondria may in part explain previous observations that a nonsecreted mutant of IGFBP3, that did not localize to the nucleus but was primarily cytoplasmic, induced apoptosis (Bhattacharyya *et al*. 2006).

Functional contacts between distinct organelles have been identified and fractionated. For example, the contact sites between ER and mitochondria are being characterized as sites for exchange of calcium (Rizzuto *et al*. 1998) and lipids (Stone & Vance 2000) between these organelles. In all, ~5–20% of the mitochondrial network surface within a cell is in close apposition to the ER (Rizzuto *et al*. 1998). We have documented the presence of IGFBP3 in MAM fraction, a subdomain of the ER, which consists of membrane tubules that provide direct physical contact between the ER and mitochondria (Vance 1990). These connections, as well as the ER and mitochondria organelles themselves, are quite variable and can undergo rapid changes in overall morphology, size, and composition (Bereiter-Hahn *et al*. 2008). Factors such as calcium homeostasis, cell metabolism, and perceived stress can have dramatic impact on both the form and function of these organelles.

In summary, we have described extranuclear trafficking of IGFBP3 and described mitochondrial and ER localization in human prostate cancer cells. IGFBP3 is well recognized to associate with cell surface membranes (Yamanaka *et al*. 1999, Mishra *et al*. 2004), and now, we have begun to characterize its association with intracellular membrane-bound organelles. Further investigation into regulation of its subcellular localization and trafficking may provide insights into the molecular mechanisms by which it induces apoptosis and provide further rationale for translation as applied to cancer therapeutics.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Bereiter-Hahn J, Voth M, Mai S & Jendrach M 2008 Structural implications of mitochondrial dynamics. *Biotechnology Journal* **3** 765–780.

Bhattacharyya N, Pechhold K, Shahjee H, Zappala G, Elbi C, Raaka B, Wiench M, Hong J & Rechler MM 2006 Nonsecreted insulin-like growth factor binding protein-3 (IGFBP-3) can induce apoptosis in human prostate cancer cells by IGF-independent mechanisms without being concentrated in the nucleus. *Journal of Biological Chemistry* **281** 24588–24601.

Bogerd HP, Fridell RA, Benson RE, Hua J & Cullen BR 1996 Protein sequence requirements for function of the human T-cell leukemia virus type 1 Rex nuclear export signal delineated by a novel *in vivo* randomization-selection assay. *Molecular and Cellular Biology* **16** 4207–4214.

Bozidis P, Williamson CD & Colberg-Poley AM 2007 Isolation of endoplasmic reticulum, mitochondria, and mitochondria-associated membrane fractions from transfected cells and from human cytomegalovirus-infected primary fibroblasts. *Current Protocols in Cell Biology* Chapter 3 Unit 3.27.

Buckway CK, Wilson EM, Ahlsen M, Bang P, Oh Y & Rosenfeld RG 2001 Mutation of three critical amino acids of the N-terminal domain of IGF-binding protein-3 essential for high affinity IGF binding. *Journal of Clinical Endocrinology and Metabolism* **86** 4943–4950.

Cao X, Liu W, Lin F, Li H, Kolluri SK, Lin B, Han YH, Dawson MI & Zhang XK 2004 Retinoid X receptor regulates Nur77/TR3-dependent apoptosis (corrected) by modulating its nuclear export and mitochondrial targeting. *Molecular and Cellular Biology* **24** 9705–9725.

Cobb LJ, Liu B, Lee KW & Cohen P 2006 Phosphorylation by DNA-dependent protein kinase is critical for apoptosis induction by insulin-like growth factor binding protein-3. *Cancer Research* **66** 10878–10884.

Cohen P 2006 Insulin-like growth factor binding protein-3: insulin-like growth factor independence comes of age. *Endocrinology* **147** 2109–2111.

Firth SM & Baxter RC 2002 Cellular actions of the insulin-like growth factor binding proteins. *Endocrine Reviews* **23** 824–854.

Fischer U, Huber J, Boelens WC, Mattaj IW & Luhrmann R 1995 The HIV-1 rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* **82** 475–483.

Gucev ZS, Oh Y, Kelley KM & Rosenfeld RG 1996 Insulin-like growth factor binding protein 3 mediates retinoic acid- and transforming growth factor beta2-induced growth inhibition in human breast cancer cells. *Cancer Research* **56** 1545–1550.

Heath-Engel HM, Chang NC & Shore GC 2008 The endoplasmic reticulum in apoptosis and autophagy: role of the BCL-2 protein family. *Oncogene* **27** 6419–6433.

Hetz CA 2007 ER stress signaling and the BCL-2 family of proteins: from adaptation to irreversible cellular damage. *Antioxidants and Redox Signaling* **9** 2345–2355.

Hong J, Zhang G, Dong F & Rechler MM 2002 Insulin-like growth factor (IGF)-binding protein-3 mutants that do not bind IGF-I or IGF-II stimulate apoptosis in human prostate cancer cells. *Journal of Biological Chemistry* **277** 10489–10497.

Jaques G, Noll K, Wegmann B, Witten S, Kogan E, Radulescu RT & Havemann K 1997 Nuclear localization of insulin-like growth factor binding protein 3 in a lung cancer cell line. *Endocrinology* **138** 1767–1770.

Jia Y, Lee KW, Swerdloff RS, Hwang D, Cobb LJ, Sinha Hikim A, Lue YH, Cohen P & Wang C 2009 The interaction of insulin-like growth factor binding protein-3 and bax in mitochondria promotes male germ cell apoptosis. *Journal of Biological Chemistry* **285** 1726–1732.

Kim FJ, Beeche AA, Hunter JJ, Chin DJ & Hope TJ 1996 Characterization of the nuclear export signal of human T-cell lymphotropic virus type 1 Rex reveals that nuclear export is mediated by position-variable hydrophobic interactions. *Molecular and Cellular Biology* **16** 5147–5155.

Lalou C, Lassarre C & Binoux M 1996 A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails to bind IGFs inhibits the mitogenic effects of IGF-I and insulin. *Endocrinology* **137** 3206–3212.

Lee KW, Liu B, Ma L, Li H, Bang P, Koeffler HP & Cohen P 2004 Cellular internalization of insulin-like growth factor binding protein-3: distinct endocytic pathways facilitate re-uptake and nuclear localization. *Journal of Biological Chemistry* **279** 469–476.

Lee KW, Ma L, Yan X, Liu B, Zhang XK & Cohen P 2005 Rapid apoptosis induction by IGFBP-3 involves an insulin-like growth factor-independent nucleomitochondrial translocation of RXRalpha/Nur77. *Journal of Biological Chemistry* **280** 16942–16948.

Lee KW, Cobb LJ, Paharkova-Vatchkova V, Liu B, Milbrandt J & Cohen P 2007 Contribution of the orphan nuclear receptor Nur77 to the apoptotic action of IGFBP-3. *Carcinogenesis* **28** 1653–1658.

Leu JI & George DL 2007 Hepatic IGFBP1 is a prosurvival factor that binds to BAK, protects the liver from apoptosis, and antagonizes the proapoptotic actions of p53 at mitochondria. *Genes and Development* **21** 3095–3109.

Li W, Fawcett J, Widmer HR, Fielder PJ, Rabkin R & Keller GA 1997 Nuclear transport of insulin-like growth factor-I and insulin-like growth factor binding protein-3 in opossum kidney cells. *Endocrinology* **138** 1763–1766.

Lithgow T, van Driel R, Bertram JF & Strasser A 1994 The protein product of the oncogene bcl-2 is a component of the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane. *Cell Growth and Differentiation* **5** 411–417.

Liu B, Lee HY, Weinzimer SA, Powell DR, Clifford JL, Kurie JM & Cohen P 2000 Direct functional interactions between insulin-like growth factor- binding protein-3 and retinoid X receptor-alpha regulate transcriptional signaling and apoptosis. *Journal of Biological Chemistry* **275** 33607–33613.

Liu B, Lee KW, Anzo M, Zhang B, Zi X, Tao Y, Shiry L, Pollak M, Lin S & Cohen P 2007 Insulin-like growth factor-binding protein-3 inhibition of prostate cancer growth involves suppression of angiogenesis. *Oncogene* **26** 1811–1819.

Malhotra JD & Kaufman RJ 2007 Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxidants and Redox Signaling* **9** 2277–2293.

Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P & Moll UM 2003 p53 has a direct apoptogenic role at the mitochondria. *Molecular Cell* **11** 577–590.

Mishra S, Raz A & Murphy LJ 2004 Insulin-like growth factor binding protein-3 interacts with autocrine motility factor/phosphoglucose isomerase (AMF/PGI) and inhibits the AMF/PGI function. *Cancer Research* **64** 2516–2522.

Moll UM, Marchenko N & Zhang XK 2006 p53 and Nur77/TR3 – transcription factors that directly target mitochondria for cell death induction. *Oncogene* **25** 4725–4743.

Radulescu RT 1994 Nuclear localization signal in insulin-like growth factor-binding protein type 3. *Trends in Biochemical Science* **19** 278.

Rajah R, Valentinis B & Cohen P 1997 Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta1 on programmed cell death through a p53- and IGF-independent mechanism. *Journal of Biological Chemistry* **272** 12181–12188.

Ricort JM, Lombet A, Lassarre C & Binoux M 2002 Insulin-like growth factor binding protein-3 increases intracellular calcium concentrations in MCF-7 breast carcinoma cells. *FEBS Letters* **527** 293–297.

Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, Tuft RA & Pozzan T 1998 Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. *Science* **280** 1763–1766.

Santer FR, Bacher N, Moser B, Morandell D, Ressler S, Firth SM, Spoden GA, Sergi C, Baxter RC, Jansen-Durr P *et al.* 2006 Nuclear insulin-like growth factor binding protein-3 induces apoptosis and is targeted to ubiquitin/proteasome-dependent proteolysis. *Cancer Research* **66** 3024–3033.

Schedlich LJ, Young TF, Firth SM & Baxter RC 1998 Insulin-like growth factor-binding protein (IGFBP)-3 and IGFBP-5 share a common nuclear transport pathway in T47D human breast carcinoma cells. *Journal of Biological Chemistry* **273** 18347–18352.

Schedlich LJ, Le Page SL, Firth SM, Briggs LJ, Jans DA & Baxter RC 2000 Nuclear import of insulin-like growth factor-binding protein-3 and -5 is mediated by the importin beta subunit. *Journal of Biological Chemistry* **275** 23462–23470.

Schedlich LJ, O'Han MK, Leong GM & Baxter RC 2004 Insulin-like growth factor binding protein-3 prevents retinoid receptor heterodimerization: implications for retinoic acid-sensitivity in human breast cancer cells. *Biochemical and Biophysical Research Communications* **314** 83–88.

Schedlich LJ, Muthukaruppan A, O'Han MK & Baxter RC 2007 Insulin-like growth factor binding protein-5 interacts with the vitamin D receptor and modulates the vitamin D response in osteoblasts. *Molecular Endocrinology* **21** 2378–2390.

Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T & Korsmeyer SJ 2003 BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. *Science* **300** 135–139.

Sell C, Rubini M, Rubin R, Liu JP, Efstratiadis A & Baserga R 1993 Simian virus 40 large tumor antigen is unable to transform mouse embryonic fibroblasts lacking type 1 insulin-like growth factor receptor. *PNAS* **90** 11217–11221.

Silha JV, Sheppard PC, Mishra S, Gui Y, Schwartz J, Dodd JG & Murphy LJ 2006 Insulin-like growth factor (IGF) binding protein-3 attenuates prostate tumor growth by IGF-dependent and IGF-independent mechanisms. *Endocrinology* **147** 2112–2121.

Spagnoli A, Torello M, Nagalla SR, Horton WA, Pattee P, Hwa V, Chiarelli F, Roberts CT Jr & Rosenfeld RG 2002 Identification of STAT-1 as a molecular target of IGFBP-3 in the process of chondrogenesis. *Journal of Biological Chemistry* **277** 18860–18867.

Stommel JM, Marchenko ND, Jimenez GS, Moll UM, Hope TJ & Wahl GM 1999 A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *EMBO Journal* **18** 1660–1672.

Stone SJ & Vance JE 2000 Phosphatidylserine synthase-1 and -2 are localized to mitochondria-associated membranes. *Journal of Biological Chemistry* **275** 34534–34540.

Sun T, Hunziker EB & Morales TI 2008 Subcellular distribution of the insulin-like growth factor (IGF) binding proteins (IGFBPs) 2 and 3 in articular chondrocytes. *Journal of Orthopaedic Research* **26** 1421–1427.

Valentinis B, Bhala A, DeAngelis T, Baserga R & Cohen P 1995 The human insulin-like growth factor (IGF) binding protein-3 inhibits the growth of fibroblasts with a targeted disruption of the IGF-I receptor gene. *Molecular Endocrinology* **9** 361–367.

Vance JE 1990 Phospholipid synthesis in a membrane fraction associated with mitochondria. *Journal of Biological Chemistry* **265** 7248–7256.

Wraight CJ, Liepe IJ, White PJ, Hibbs AR & Werther GA 1998 Intranuclear localization of insulin-like growth factor binding protein- 3 (IGFBP-3) during cell division in human keratinocytes. *Journal of Investigative Dermatology* **111** 239–242.

Yamamoto H & Murphy LJ 1994 Generation of des-(1–3) insulin-like growth factor-I in serum by an acid protease. *Endocrinology* **135** 2432–2439.

Yamamoto H & Murphy LJ 1995 Enzymatic conversion of IGF-I to des(1–3) IGF-I in rat serum and tissues: a further potential site of growth hormone regulation of IGF-I action. *Journal of Endocrinology* **146** 141–148.

Yamanaka Y, Fowlkes JL, Wilson EM, Rosenfeld RG & Oh Y 1999 Characterization of insulin-like growth factor binding protein-3 (IGFBP-3) binding to human breast cancer cells: kinetics of IGFBP-3 binding and identification of receptor binding domain on the IGFBP-3 molecule. *Endocrinology* **140** 1319–1328.

Yan X, Forbes BE, McNeil KA, Baxter RC & Firth SM 2004 Role of N- and C-terminal residues of insulin-like growth factor (IGF)-binding protein-3 in regulating IGF complex formation and receptor activation. *Journal of Biological Chemistry* **279** 53232–53240.